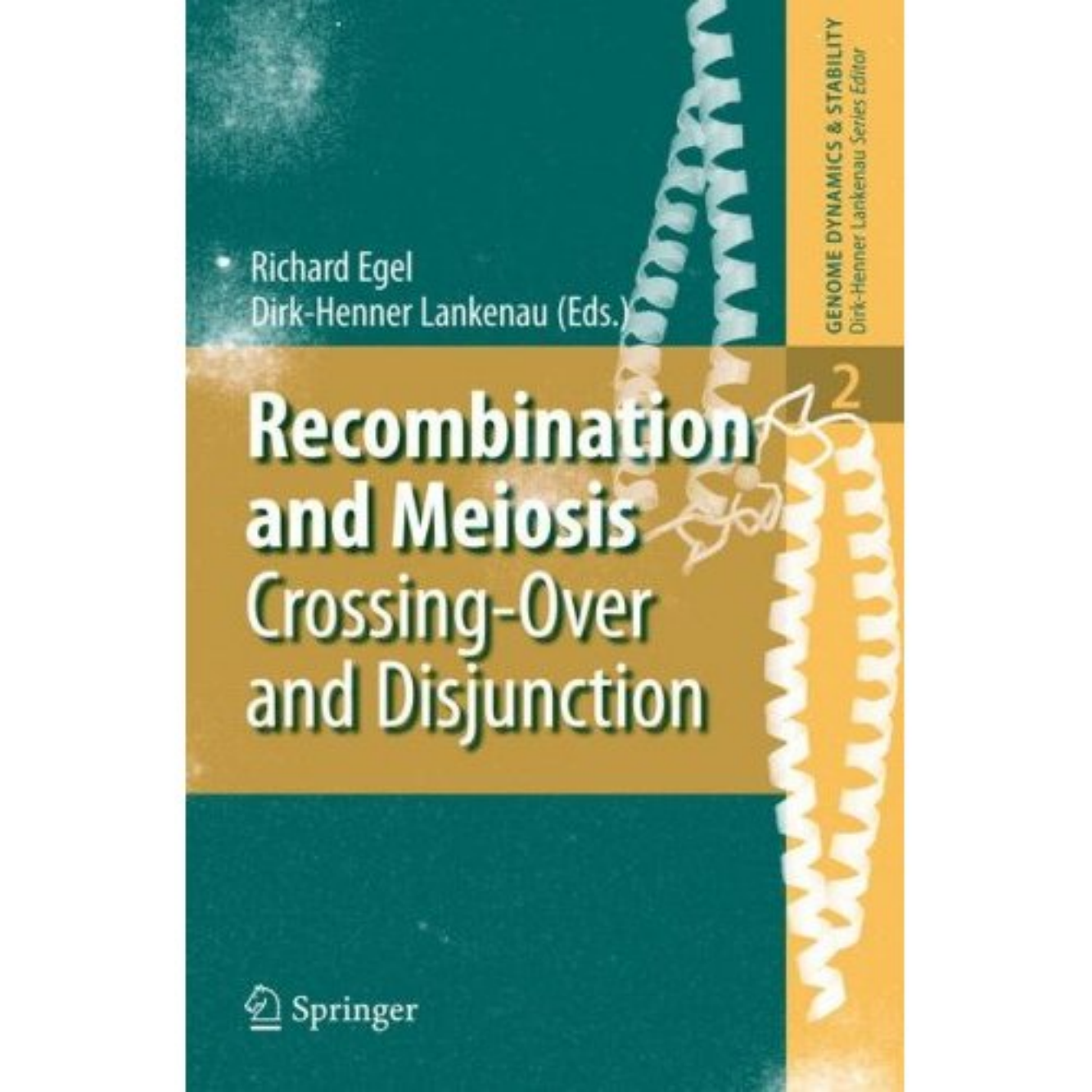


The background of the book cover features an abstract representation of DNA and chromosomes. A teal-colored section at the top and bottom contains a faint, glowing DNA double helix. A central orange band contains a more prominent, stylized white DNA double helix that appears to be in motion or interacting with other structures. The overall design is scientific and modern.

• Richard Egel
Dirk-Henner Lankenau (Eds.)

GENOME DYNAMICS & STABILITY
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Recombination and Meiosis

Crossing-Over and Disjunction



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Recombination and Meiosis

Crossing-Over and Disjunction

Volume Editors: Richard Egel, Dirk-Henner Lankenau

With 47 Figures

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Cover

The cover illustration depicts two key events of DNA repair: 1. The ribbon model shows the structure of the termini of two Rad50 coiled-coil domains, joined via two zinc hooks at a central zinc ion (sphere). The metal dependent joining of two Rad50 coiled-coils is a central step in the capture and repair of DNA double-strand breaks by the Rad50/Mre11/Nbs1 (MRN) damage sensor complex. 2. Immunolocalization of histone variant γ -H2Av in γ -irradiated nuclei of *Drosophila* germline cells. Fluorescent foci indicate one of the earliest known responses to DNA double-strand break formation and sites of DNA repair.

(provided by Karl-Peter Hopfner, Munich and Dirk-Henner Lankenau, Heidelberg)

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Preface

Volume 1 of the current series on *Genome Dynamics and Stability* identified *Genome Integrity* as the *non plus ultra* requirement for cellular life. Whether it is extracellular viral genomes, cellular prokaryotic or eukaryotic genomes, the integrity of genomes is the precondition for all life. This criterion is reflected in the underlying biochemical DNA/RNA metabolism processes, mainly represented by DNA/RNA replication/transcription and DNA repair. We now present the second book of this series. It deals with *Recombination and Meiosis: Crossing-Over and Disjunction*. It will soon be accompanied by a third book, likewise dealing with recombination and meiosis, but focusing a little more on theory–practice coupled approaches. The title of the third book will be: *Recombination and Meiosis: Models, Means and Evolution*.

When cells, during evolution, assembled into multicellular aggregates – a phenomenon we have to accept as a fact of complex life that has happened more than once – many of the most basic genome-maintenance factors were reshaped by Darwinian selectional forces. To be sure, long before the emergence of multicellular organisms, cyclic mechanisms became established to combine two haploid genomes and to reduce the diploid genome back to haploid ones. Yet, the relative abundance of haploid versus diploid stages remained highly variable. After billions of years of unicellular evolution, within a lineage stemming from a diploid protist with gametic meiosis, the origin of modern metazoans began in a (pre)cambrian diversification (i.e. explosion) to multicellular diversity where selectional forces always had a broad spectrum of molecular factors, phenomena and mechanisms to act upon. Among the molecular and cellular key processes making multicellular complexity possible were i) the potentially immortal germline from which somatic cells differentiate and ii) meiosis to precisely half the number of chromosomes established in the zygote. The differentiation of gametes into resourceful, immobile eggs and highly motile sperm cells probably developed very early in the metazoan lineage. In a certain, evolutionarily meaningful, way the animal body can be considered the germ cells' most successful means of being nourished and disseminated.

As a cytogenetic phenomenon preceding gametogenesis, where homologous chromosomes undergo programmed crossing-over and recombination, meiosis has been known since the early days of the chromosome theory of inheritance, but only more recently have the underlying molecular processes

become accessible. The present book focuses on crossing-over between and disjunction of chromosomes during the meiotic cell cycle.

The first chapter is an introductory overview written by Richard Egel, the initiator of this twin-volume edition; this synopsis covers the scope of both accompanying books. The second chapter by José Suja and Julio Rufas deals with the highly condensed cores of mitotic and meiotic chromosomes, their supramolecular structures and the involved segregation processes. Written by these leading specialists on visualizing the core structures by silver staining, it presents the current view on the relationship between the chromatid cores and the synaptonemal complex lateral elements, DNA topoisomerase II α , and the glue between individual chromosomes, i.e. condensin and cohesin complexes, is assessed. The third chapter is written by Koichi Tanaka and Yoshinori Watanabe. It represents pioneering work in unraveling the molecular systems of chromatid cohesion. We are here confronted with key questions as to how mono-oriented sister kinetochores attach to microtubules, each to only one cellular pole, and how sister chromatids separate during meiosis I, while homologs remain paired until their segregation in meiosis II. The centrally important key proteins are presented. The fourth chapter is written by another pioneer, Scott Keeney, who discovered the DNA double-strand break (DSB) initiating Spo11 protein in yeast and the mechanism involved in how chromosomes initiate programmed recombination during meiosis by means of this archaeal-like topoisomerase. The fifth chapter by Sonam Mehrotra, Scott Hawley and Kim McKim deals with *Drosophila* as a metazoan model organism providing molecular, genetic and cytological details on how meiotic pairing and synapsis can proceed independently of programmed DSBs in DNA. Further, it elucidates the relationship of DSB formation to synapsis, how crossovers are determined and formed, and the role of chromosome structure in regulating DSB formation and repair, including specialized pairing sites. The chapter by Terry Ashley deals with recombination nodules in mammalian meiotic chromosomes and the dynamics of shifting protein compositions, while cytological structures remain nearly constant. The seventh chapter by Celia May, Tim Slingsby and Sir Alec Jeffreys exploits the human HapMap project to shed light on recombinational hot spots in human chromosomes during meiosis. The eighth chapter by Haris Kokotas, Maria Grigoriadou and Michael Petersen reviews our current understanding of human chromosomal abnormalities, as caused by meiotic nondisjunction, using Trisomy 21 as a case study.

While metazoans dominate the chapters so far – with some recourse to yeasts – plants represent another multicellular kingdom of life. In the ninth chapter Gareth Jones and Chris Franklin focus on botany's most prominent model system, i.e. *Arabidopsis thaliana*. It reviews meiotic recombination, chromosome organization and progression in this model plant, which of course, stands in for the key role of plants in agricultural production. Finally, Livia Pérez-Hidalgo, Sergio Moreno and Christina Martin-Castellanos link the meiotic program to modified aspects of mitotic cell cycle control. It reviews how mitotic regulators

adapt or are co-opted to the functional necessities of the meiotic program, paying particular attention to meiosis-specific factors whose functions are essential for meiosis. This comparative review is rooted in the pioneering cell-cycle studies on baker's yeast (*Saccharomyces cerevisiae*) and fission yeast (*Schizosaccharomyces pombe*), from where it extends to mammalian gametogenesis and other multicellular eukaryotes. A similar range of model studies has also applied to the scope of the chapter by Tanaka and Watanabe and the review of Scott Keeney.

Following the contents table of this book, the list of forthcoming chapter titles in the accompanying volume is included in advance. In fact, as some of the individual chapters had been published online first, before the editorial decision to divide the printed edition into two books was taken, the preliminary cross-references had not yet accounted for the split. We apologize for any inconvenience this may cause, but the listing of all the chapter titles in both books should hopefully direct the reader to the proper destination. We would also like to point out that the missing chapter numbers are not neglect but reflect an obligatory compromise necessitated by publishing all the manuscripts OnlineFirst immediately after they have been peer reviewed, revised, accepted and copy edited (see, <http://www.springerlink.com/content/119766/>).

We most cordially thank all the chapter authors for contributing to this topical edition of two accompanying books. Without their expertise and dedicated work this comprehensive treatise would not have been possible. Receiving the incoming drafts as editors, we had the great privilege of being the first to read so many up-to-date reviews on the various aspects of meiotic recombination and model studies elucidating this ever-captivating field. Also, we greatly appreciate the productive input of numerous referees, who have assisted us in striving for the highest level of expertship, comprehensiveness and readability.

We are also deeply indebted to the Springer and copy-editing staff. In particular, we would like to mention Sabine Schreck, the editor at Springer Life Sciences (Heidelberg), Ursula Gramm, the desk editor (Springer, Heidelberg), and Martin Weissgerber, the production editor (LE-TeX GBR, Leipzig).

Copenhagen,
Ladenburg, July, 2007

Richard Egel
Dirk-Henner Lankenau

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Meiotic Crossing-Over and Disjunction: Overt and Hidden Layers of Description and Control

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Abstract Sexual reproduction is observed in the vast majority of eukaryotic organisms. Foremost, this includes animals, plants, and fungi. In the course of sexually propagated generations, the regularities of Mendelian genetics and the segregation of partly recombined chromosomes at meiosis are two complementary faces of one and the same coin. This chapter opens the first book of two in a series, both volumes being dedicated to the complex process of meiotic recombination. This editorial synopsis focuses on the various facets of meiosis from a descriptive perspective, before the specific chapters discuss the details of molecular mechanisms. Meiosis and mitosis are viewed as alternative schemes of eukaryotic chromosome segregation, which supposedly have coevolved from a very early start. The structure and kinetics of meiotic bivalents depend on the formation of chiasmata between non-sister chromatids and the different stability of sister-chromatid cohesion along the chromosome arms and at the centromeres. The relevance of spindle dynamics for bivalent segregation and potential nondisjunction is discussed. Telomere clustering plays an assisting role during the intermediate phase of the bouquet arrangement. At the heart of meiotic prophase, pairing and synapsis of homologous chromosomes is accompanied by genetic crossing-over and chiasma formation. The what, where, and how of DNA exchange proceed from site facilitation via partner choice and homology search to the formation and resolution of heteroduplex intermediates. The nonrandom distribution of crossovers and chiasmata is subject to interference mechanisms at various levels. Finally, the segregation of chromosomes during meiosis I and II is accomplished by an interplay of basically mitotic proteins with meiosis-specific components.

Abbreviations

DSB	double-strand break
ds/ssDNA	double-/single-stranded DNA
HR	homologous recombination
MTs	microtubules
K-fiber	MT nucleated at the kinetochore
SC	synaptonemal complex

1

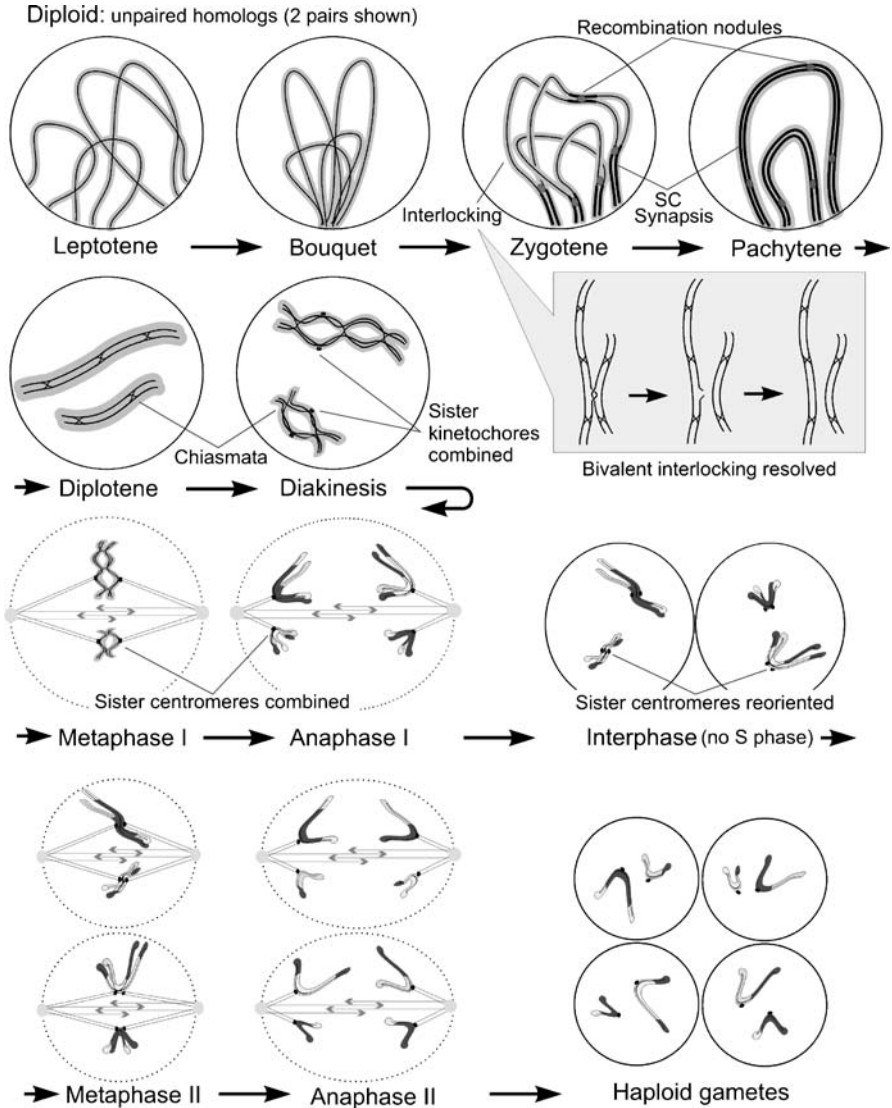
Characteristics of Meiotic Segregation*Intricate duplicity reduced*

The life cycle of sexually propagating organisms alternates between two modes of nuclear division, mitosis and meiosis. While mitosis is the “working horse” of identical cell proliferation, usually repeating itself for many divisions in a row, meiosis has more exclusive rights, just once per life cycle (Sect. 2). Also, meiosis requires diploid cells, takes more time, and is more complicated at various levels; thus meiosis is more difficult to describe in straightforward and yet unambiguous terms. As in mitosis, meiosis is preceded by a round of DNA synthesis, but this single replication is followed by two rounds of chromosome segregation and nuclear division in a row – meiosis I and II. Uniquely to mainstream meiosis, a major part of the long-lasting meiotic prophase is devoted to intricate pathways of genetic recombination and chiasma formation, before the reshuffled chromosomes are segregated in two rounds. There are two main components to the meiotic redistribution of genetic material. (i) The parental chromosomes, as defined by their centromeres, are reassorted independently. (ii) The parental gene combinations on the chromosomal arms are further scrambled by crossing-over, the number position of which can vary from meiosis to meiosis. The overall result leads to four haploid postmeiotic nuclei, reducing the ploidy by half (Fig. 1).¹

Fig. 1 Main stages of meiosis. *Leptotene*: Axial cores are visible along the chromosomes; sister chromatids are still intimately united. *Bouquet* arrangement: All the telomeres are clustered in a narrow region at the inner membrane of the nuclear envelope. *Zygotene*: Synaptonemal complexes (SC, marking homolog synapsis) are initiated at terminal and/or interstitial nucleation points. Recombination nodules appear, marking sites of potential chiasmata. Topological interlocking of two or more bivalents is not infrequent. *Inset*: To resolve an interlock, one of the axial cores must be broken (i.e., both sister chromatids). After the entrapped bivalent has escaped, the double-gap must be sealed, probably facilitated by SC closure. *Diplotene*: SCs disintegrate, individual chromatid cores become visible close to chiasmata. *Diakinesis*: Homologs separate, except at chiasmata; chromatid cores separate along the chromosomes, except at the centromeres. *Meta-/Anaphase I*: Fused sister kinetochores segregate to the same pole to separate the bivalents; outer chromatid arms are partly recombined. *Interphase*: There is no S phase; sister kinetochores reorient to opposite sides of each chromosome. *Meta-/Anaphase II*: Sister kinetochores segregate to opposite poles, thus producing four haploid gametes

¹ This introductory chapter provides a synoptic view over the entire field and the topical chapters to follow, with no intention of duplicating the many references to original work cited therein. Cross-references to other chapters in this volume are cited as “this BOOK” or, if placed in the accompanying volume, as “this SERIES” (see extended “Table of Contents” preceding this chapter).

In this chapter, the description of meiotic mechanisms is focused on two main components: the transient reorganization of centromeres and the reshuffling of chromosome arms by chiasmata. In certain deviations from the mainstream regimen, one of these aspects can be observed without the other, which can make the task of an unambiguous description less difficult. The classical model organism of formal genetics, the fruit fly *Drosophila melanogaster*, follows the mainstream pattern only in female meiosis, whereas the males perform spermatogenesis without genetic



crossing-over or chiasma formation (achiasmatic disjunction).² Hence, the simplified version of achiasmatic meiosis is presented first.

1.1

Kinetic Activity at the Centromeres

Splitting the deal

In general, the occurrence of meiosis before the formation of germ cells serves two major objectives, the halving of chromosome number and the reshuffling of chromosomal gene contents. How is the halving by number accomplished in the achiasmatic meiosis of *Drosophila* males?

Before all the chromosomes can be disjoined in order, the pairs of homologs must physically communicate. In male meiosis this is solely accomplished by interconnections at special pairing sites³ (McKee 1998), which appear to require transcription to be active. Two meiotic proteins have been shown to be involved in this conjunction, one being related to cohesin proteins (Thomas et al. 2005). These connections have to persist until metaphase of meiosis I. At the crucial steps of metaphase and anaphase it is important that the centromeres are organized differently in meiosis I as compared to mitosis, in that sister kinetochores are fused as a functional unit (J.A. Suja and J.S. Rufas, this BOOK). This is the same in male and female meiosis of *Drosophila*. In consequence, both sister kinetochores attach to the same spindle pole, and the kinetochores of the connected homolog attach to the other pole. At anaphase I, therefore, sister kinetochores are drawn to the same pole; both sister chromatids of each chromosome thus stay together entirely and are separated from both chromatids of the homolog.

In the short interphase between meiosis I and II, the centromeres reorganize so that sister kinetochores again are separated and face in opposite directions, as in mitosis. In consequence, they attach to spindle fibers from opposite poles, and the sister chromatids with all their genes then segregate from one another at anaphase II. The latter condition, in particular, no longer holds for chiasmatic meiosis, where the sister chromatids are broken up and scrambled by reciprocal exchange between the homologs.

² Another form of achiasmatic meiosis occurs in oocytes of the silkworm *Bombyx mori*, where a modified synaptonemal complex (Sect. 5) ensures the stabilization of bivalents until metaphase I. Significantly, if chiasmatic meiosis is restricted to one gender only, it is usually the “heterogametic” gender that no longer undergoes crossing-over and chiasma formation, such as in XY-bearing *Drosophila* males and WZ-bearing *Bombyx* females. This differential suppression of crossing-over has likely resulted from selection against recombinational rearrangements between the diverged sex chromosomes.

³ Similar pairing sites may also be involved the early stages of chiasmatic meiosis of *Drosophila* females or other organisms (Sect. 5), but their influence does not usually persist until metaphase.

1.2

The Structural Relevance of Chiasmata

Sister ain't your sister, but ...

The chiasmata observed in mainstream meiosis serve both a genetic purpose (Sect. 8) and a structural role for the segregation mechanism itself. Without chiasmata, the paired up homologs (termed bivalents) would fall apart before metaphase. Each individual chromosome would then be free to attach to either spindle pole, independently of its homolog⁴, approaching a 50% risk of “nondisjunction”, when both coincidentally are gathered at the same pole (H. Kokotas, M. Grigoriadou and M.B. Petersen, this BOOK). The structural glue that manifests itself by bivalent stability in the presence of chiasmata can be ascribed to sister-chromatid cohesion (K. Tanaka and Y. Watanabe, this BOOK), notably in the distal parts of the chromosome arms, facing away from the centromeres (J.A. Suja and J.S. Rufas, this BOOK).

This is a formidable challenge to a fairly common mantra of meiosis, that *sister chromatids stay together in meiosis I, only to be separated equationally in meiosis II*. While this description, in fact, is fully valid for achiasmatic meiosis (Sect. 1.1), it no longer fits unconditionally for the mainstream form of chiasma-based meiosis (Fig. 1). To save the relevant part of the commonly repeated phrase, and do justice to the fundamental importance of meiotic chiasmata as well, it is necessary to observe the following qualifications.

With due consideration of the local constraints imposed by the chiasma, the said notion can still be applied to the sister kinetochores themselves and the adjacent segments of sister chromatids, up to the first chiasma on either side. For these innermost parts alone, disjunction at meiosis I will always be reductional. For the next segments, between the first and the second chiasma, sister chromatids are always segregated in meiosis I already. Yet, further out beyond the second chiasma, it will be 50 : 50 whether sister chromatids separate in meiosis I or II. Ironically, therefore, where sister-chromatid cohesion is most important for bivalent stability in metaphase I (just distal of the first chiasma from the centromere), these parts of sister chromatids will *never* stay together in anaphase I. On average, therefore, only half the genes in the genome will follow the segregational pattern laid out by the centromeres, that *sister kinetochores stay together in meiosis I, only to be separated equationally in meiosis II*; the other half will just do the opposite.

Sister chromatid cohesion is critical in providing the structural support for bivalent stability at metaphase. It balances the pulling forces exerted by spindle fibers towards the spindle poles (Sect. 9). Eventually, though, this cohesion must dissolve, thus giving way to the segregational movements at

⁴ In several organisms, recombination-independent centromere association can still favor proper homolog disjunction to some extent (Davis and Smith 2003; see D.Q. Ding and Y. Hiraoka, this SERIES).

anaphase. This release is mediated by proteolytic cleavage of a connecting subunit in the cohesin complex. As a characteristic modification at meiosis, the release of sister chromatid cohesion occurs in several steps, for different parts of the chromosomes (K. Tanaka and Y. Watanabe, this BOOK). At first, at the metaphase/anaphase transition of meiosis I, it is only dissolved along the arms. This releases the topological constraints at the chiasmata where the partly exchanged chromatids had been physically interlaced. Around the centromeres, however, the cohesin complexes remain intact until they are dissolved at the metaphase/anaphase transition of meiosis II.

Other structural changes concern the topology of so-called chromatid cores, which form the connecting threads in a radial-loop/scaffold model of chromatin organization in chromosomes (J.A. Suja and J.S. Rufas, this BOOK). These scaffolding cores consist of various proteins, such as topoisomerase II and condensin⁵ complexes, respectively involved in the decatenation of interlocking DNA loops and the successive contraction of chromatid arms in the preparation for division. Very characteristically, the contraction of sister chromatids appears to proceed by “relational coiling”, giving opposite helical handedness to both strands. This may effectively pry the sister chromatids apart until fewer and fewer interlocks remain to be resolved by the topoisomerase. As meiotic prophase proceeds beyond the stage of homolog synapsis (Sect. 5), the chromatid cores separate first at the sites of chiasmata. At this stage it becomes evident that a seamless reconnection has been established at the light-microscopic resolution of chromatin superstructure, reflecting the molecular exchange of the corresponding DNA molecules by a genetic crossover event. This reconnection of chromatid cores at chiasma sites is likely prepared by the so-called recombination nodules, which can be visualized by electron microscopy (and/or immunostaining for specific protein components) even at the preceding synapsis stage (T. Ashley, this BOOK).

2

The Staging of Meiosis

The ultimate alternative

The genetic exchange with matching partner chromosomes, as observed in mainstream meiosis, requires matching pairs of homologs to begin with. For a primarily haploid unicellular organism, this means that two haploid cells have to merge and combine their nuclear genomes before meiosis can commence to rearrange both sets of chromosomes.

⁵ Condensin proteins are structurally related to the cohesins mentioned before, but the mechanisms of their action and control are not yet fully explored.

2.1

Life-Cycle Variants

The purely haplontic life cycle likely represents an early setting in eukaryote evolution (R. Egel and D. Penny, this SERIES). It is characterized by vegetative propagation of haploid single cells, and meiosis occurs in zygotes (zygotic meiosis), the fusion nucleus being the only diploid stage in the life cycle. Among extant eukaryotes, however, this simple scheme is not observed abundantly. Three scattered examples of this category are the social amoeba *Dictyostelium discoideum*, the unicellular green alga *Chlamydomonas reinhardtii*, and the fission yeast *Schizosaccharomyces pombe*. In these, meiosis is related to the formation of dormant resting stages, zygotic cysts in the first two cases and ascospores in the third example.

In contrast, gametic meiosis prevails in the purely diplontic life cycle of metazoans, immediately before the formation of dimorphic gametes, the female eggs, and the male spermatocytes. Accordingly, these gametes are the only haploid cells occurring in either gender, and the diploid phase is reestablished upon fertilization by sperm/egg fusion. The fertilized egg, or zygote, develops into various lines of stem cells, from which the differentiated body tissue cells derive. Typically, it is only the most universal class of stem cells that ultimately can lead to meiosis anew, thus giving rise to the next generation of germ cells. What it is at the molecular level that sets the so-called germline apart from ordinary soma cells is still under active investigation (D.-H. Lankenau, this SERIES).

In addition to the purely haplontic or diplontic extremes, a varied spectrum of mixed strategies unfolds in other organisms, where meiosis and fertilization are separated by mitotic cell divisions both at the haploid and the diploid level. Even though flowering plants (e.g., *Arabidopsis thaliana*, G.H. Jones and F.C.H. Franklin, this BOOK) superficially resemble the diplontic cycle of animals, the evolutionary history relates their breeding system to alternating generations of diploid “sporophytes” and haploid “gametophytes”. Yet, while both these generations can comprise many somatic cell divisions in algae, mosses⁶ or ferns, the haploid gametophytes of flowering plants have been reduced to inconspicuously few nuclear divisions that are well hidden within the “female” flower parts of their diploid host plants⁷, where seed formation is initiated.

⁶ In mosses and horn-worts, the life cycles are actually dominated by the habitus of the haploid gametophyte stage.

⁷ In this nomenclature, all the visible parts of a flowering plant belong to the diploid sporophyte, which produces two kinds of haploid meiospores. The microspores or pollen grains adopt the male role in cross-fertilization, and the megaspores adopt the female role. While the megaspores of modern plants develop into fertilizable ovules directly, the microspores germinate to form a pollen tube (the male gametophyte) with two or more haploid nuclei, only one of which fuses with the ovular nucleus during fertilization.

In the third kingdom of multicellular eukaryotes, the filamentous fungi, the uncoupling of meiosis from cellular or hyphal fusion leads to yet more diverse variation, in that nuclear and cytoplasmic phases are essentially uncoupled. As to their nuclear state, most fungi actually follow a strictly haplontic cycle, where meiosis proceeds directly from karyogamy, the sexual fusion of two haploid nuclei. This is then followed by the formation of haploid spores.⁸ Notably, the ultimate fusion of nuclei before meiosis is preceded by extended periods of vegetative growth where two types of haploid nuclei share a common cytoplasm.⁹ The characteristic fruiting bodies of mushrooms belong to this category. At this stage, complementary gene functions can be expressed in the common cytoplasm, even though the individual nuclei remain genetically distinct and haploid. Only rather few fungi have developed regular stages of diploid growth, such as the infectious phase of plant-pathogenic smut fungi (e.g., *Ustilago maydis*) or the unicellular bakers yeast (*Saccharomyces cerevisiae*).

In addition to the multicellular members of the so-called crown group (comprising animals, fungi, and green plants) there are the numerous phyla of unicellular protists. Some of these add further variety to life-cycle strategies, and many others are not fully explored in that respect. Arguably the most interesting and complex variation is found in ciliates (such as *Tetrahymena* or *Paramecium*), which at the unicellular level operate with dimorphic nuclei of different function (see Katz 2001). Of these, transcription for protein synthesis is limited to the highly polyploid macronucleus, which typically can only last for a certain number of vegetative cell divisions. On the other hand, the diploid micronucleus is dedicated to a merely generative role. During the sexual encounter of ciliate conjugation the macronuclei are resorbed, and only the micronuclei of both partners undergo meiosis. Three of four postmeiotic nuclei are resorbed as well, and the remaining one divides at least once at the haploid level. Each conjugant cell retains one of these nuclei and exchanges the other with its partner, and the respective nuclei fuse and divide mitotically at the diploid level. Thereafter, one of the diploid nuclei is retained as the new micronucleus, and the other one regenerates the new macronucleus. In operational terms, this nuclear division of labor very much resembles the germline/soma differentiation observed in multicellular metazoans.

⁸ The fungal meiospores can be formed inside a larger cell (the ascospores of ascomycetes) or be extruded from a basal cell (the basidiospores of basidiomycetes).

⁹ If the sexually different nuclei associate in pairs and divide coordinately in a stereotype pattern of retrograde migration of one of the daughter nuclei, this mixed phase is called a dikaryon; otherwise, if several nuclei are contained in syncytial mycelia without pairwise coordination, this is referred to as a heterokaryon.

2.2

Cell-Cycle Reprogramming

Within the various life-cycle strategies, individual cells are programmed ahead of time as to whether their next division will follow the mitotic pattern by default, or will prepare for meiosis instead (L. Pérez-Hidalgo, S. Moreno and C. Martín-Castellanos, this BOOK). In metazoans, the entry into meiosis of germline cells is largely controlled by specialized somatic cells, such as Sertoli cells in the testicle and follicle cells in the ovary, which nurture the developing germ cells through spermatogenesis and oogenesis, respectively. In potentially immortal, unicellular organisms, however, essentially all the cells are able to switch to the sexual program of cell fusion and/or meiosis, in due response to appropriate environmental signals. Comparing different organisms in terms of cell cycle regulation in this regard, transcription factors have been recruited anew or decommissioned many times, so the contribution of regulatory components has been conserved rather poorly during evolution. Hence, I will only highlight certain superior principles for this synoptic view.

By and large, meiosis and mitosis stand out as two modular alternatives for a single cell to organize its next division. Even though numerous functional components are common to both mitosis and meiosis, others are not, and the specific ones are usually subject to multiple control systems. In brief, here are some informative examples for molecular toggle switch systems, which ensure the mutually exclusive performance of either program.

In nematodes (the roundworm *Caenorhabditis elegans*), two antagonistic signal sets direct developing germline cells towards mitosis in the beginning, or towards meiosis later on (Kimble and Crittenden 2005; Suh et al. 2006). The mitotic set comprises a Notch-type¹⁰ membrane receptor and several RNA-binding proteins. The stimulating Notch signal originates from a single somatic cell at the tip of the developing gonad, and its strength diminishes with distance from the source. The meiosis-promoting set, on the other hand, comprises both a transcriptional and a translational repressor, a cytoplasmic poly(A) polymerase, and another RNA-binding protein. Notably, each set of regulatory factors downregulates expression of the other set. Accordingly, mitotic proliferation of germline cells prevails close to the tip cell, and meiosis is initiated in a sliding zone from the other end of the gonad. Still, among mRNAs to be controlled, the most important downstream targets that react to these signals remain to be identified.

As to free-living yeasts, every single cell is potentially capable of entering meiosis, which then is followed by ascospore formation. This occurs in response to a combination of internal and environmental signals (L. Pérez-Hidalgo, S. Moreno and C. Martín-Castellanos, this BOOK). Both fission yeast (*S. pombe*) and bakers yeast (*S. cerevisiae*) need to be heterozygous

¹⁰ A widely conserved intercellular signalling pathway named after the *Drosophila* Notch mutant.

for mating-type genes, which ensures that only diploid cells can engage in meiosis and sporulation. Furthermore, the nutrient supply from the medium should be depleted, especially for a nitrogen source. In both these yeasts, the induction of meiosis depends on upregulation by critical (though nonhomologous) transcription factors, *Ste11* in *S. pombe* and *Ime1* in *S. cerevisiae*. Also, in *S. pombe*, a protein kinase (*Pat1*) that normally inactivates all meiotic activities in the vegetative state has to be specifically inactivated first (reviewed by Yamamoto 2004). This extra safeguard has not been observed in baker's yeast; it may be related to the predominantly haploid mode of fission yeast cells, where the inadvertent induction of meiosis would be especially precarious.¹¹

Transcriptional regulation has long been considered key to understanding how the cell division machinery is switched from the ordinary mitotic mode to the meiotic alternative. Indeed, large sets of genes are preferentially expressed during meiosis, as shown by genome-wide analyses in both *S. cerevisiae* and *S. pombe* (Chu et al. 1998; Mata et al. 2002). Yet, the either/or of this bifurcation is also corroborated at other levels of control, such as differential mRNA stability (Daga et al. 2003), alternative splicing of meiotic transcripts (Juneau et al. 2007) or meiosis-specific translational control (Reynolds et al. 2007).

Studies in both model yeasts suggest that the decision to initiate meiosis has to be taken before "premeiotic" S phase. This makes DNA synthesis an integral part of the meiotic program of molecular events. What then is special about this crucial round of replication? From studies on meiosis in lily anthers it was deduced that replication of some DNA ($\gg 1\%$) was delayed from general S phase to zygotene (Hotta et al. 1985). This special DNA could then have played a role in homolog pairing and synapsis. Yet, similar findings have not since been extended by others to other organisms; so the generality of this assumption remains unproven. On the other hand, premeiotic DNA synthesis need not be different as such, if only the critical processes happened to be associated with S phase. This could be the loading of ancillary protein complexes, such as meiosis-specific cohesins. As to mitotic cohesins, it has indeed been shown that sister-chromatid cohesion is established at replication forks, after the necessary loading of cohesin complexes has occurred before S phase (Uhlmann and Nasmyth 1998; Lengronne 2006).

Later on, the direct succession of meiosis I and II (without an intervening round of DNA replication) requires a delicate balancing of cyclin-dependent protein kinases and other regulatory factors (L. Pérez-Hidalgo, S. Moreno and C. Martín-Castellanos, this BOOK). Moreover, the special features of meiotic prophase concerning homolog pairing, synapsis, and recombination are discussed in the following sections.

¹¹ Starting meiosis from the haploid state, of course, has detrimental consequences and is avoided by special safeguarding controls; conditionally lethal *pat1^{ts}* mutants can be obtained in *S. pombe*, which initiate meiosis at the nonpermissive temperature.

3

The Essence of Meiotic Recombination and Marker Exchange

Shuffling the deck

Next to reducing by half the number of chromosomes from the diploid level, the hallmark of meiosis is the exchange of genetic markers, inherited from slightly different parents. This can be due to the random assortment of non-homologous chromosomes in meiosis I, as well as the breakage and rejoining of homologs in meiotic prophase.¹² Among the various types of genetic recombination (Box 1; J.E. Haber, this SERIES), reciprocal exchange events between homologs are especially important, since only these produce chiasmata. More detailed analyses have suggested that essentially every meiotic crossover event is associated with the formation of some heteroduplex DNA at the actual site of molecular recombination (Borts and Haber 1987). This can result in a limited segment of nonreciprocal recombination (gene conversion and/or postmeiotic segregation), together with the reciprocal exchange of all the other markers that lie outside and are not involved in heteroduplex formation. In addition, there are other events of local heteroduplex formation that do not lead to chiasmata. Such events can still be observed as a limited stretch of gene conversion, with no reciprocal exchange of outside markers.

Box 1 Glossary: Basic terms relating to genetic recombination

Recombinants	Progenies in which markers from different parents are recombined.
Assortment of chromosomes	Independent segregation of different parental chromosomes in meiosis I. Genetic recombinants can arise without the molecular recombination of DNA.
Crossing-over	Reciprocal exchange of linked genetic markers. Both types of recombinants can be recovered from the same meiosis (usually by tetrad analysis).
Gene conversion	Nonreciprocal exchange of linked genetic markers, most commonly observed as 3 : 1 segregation of two alleles in tetrad analysis.
Post-meiotic segregation	Segregation of genetic markers in the first mitosis after meiosis II, most commonly observed as 5 : 3 segregation of two alleles in extended tetrad analysis. This is attributed to the formation of heteroduplex DNA as a recombinational intermediate.

¹² Thus, inasmuch as nonallelic genetic markers are carried on different chromosomes, recombinant progeny can also result from achiasmatic meiosis. For markers on the same chromosome, however, recombinants can only arise from crossing-over and chiasmata.

Box 1 (continued)

Aberrant 4 : 4 segregation	Presence of heteroduplex DNA in two chromatids of a meiotic tetrad, as inferred from post-meiotic segregation of the same allelic markers twice, in parallel mitotic divisions after meiosis II.
Chiasma	The cytologically visible result of a crossover event.
Homolog pairing	Approximate alignment of homologous chromosomes
Synapsis	Tight juxtaposition of homologs, mediated by synaptonemal complex (SC) structures
Recombination nodule	Electron-dense structure correlating with recombination events, usually associated with SCs
Bivalent	Pair of homologous chromosomes, connected by SC or chiasmata
Univalent	Single chromosome lacking chiasmata or a pairing partner

In most organisms, meiotic crossing-over can occur along most of the chromosomes, with the exception of certain cold-spot regions, such as pericentromeric heterochromatin and the rDNA repeats of the nucleolar organizer. Upon closer inspection, the actual exchange point distribution throughout the euchromatic regions is not entirely uniform, but is often marked by distinctive peaks of recombinational hotspots (C. May, T. Slingsby and A.J. Jeffreys, this BOOK). Some of these hotspots may be due to preferential sites of DNA breakage, but preferential resolution of other recombinational intermediates may also be involved.

4**The Enigma of Partner Choice**

Welcome, Parvenu!

Essentially all the major players in the molecular pathways to meiotic crossing-over are either identical with enzymes involved in recombinational repair in mitotic cells, or evolutionarily related to such activities (W.D. Heyer, this SERIES). Yet, the “damage” that needs repair, and thus triggers meiotic recombination, is by no means accidental. In contrast to endogenous or environmental DNA damage, which may hit any cell at any time, the double-strand breaks (DSBs) that appear to be required for meiotic crossing-over are catalyzed by a special enzyme that has no other function in the life cycle of the organism. First discovered in yeast, the Spo11 family of proteins is homologous to topoisomerase VI from Archaea. Differently from ordinary endonucleases, a Spo11 dimer does not leave its substrate after the reaction,

but remains covalently attached to the 5' ends at either side of the break (S. Keeney, this BOOK). Hence, the cut DNA is not subject to unrestrained extension of the damage, but is directly processed further by one of several repair pathways in a carefully controlled fashion.

When a diploid mitotic cell, at G₂ of the cell cycle¹³, suffers DNA breakage, it has several choices for staging a templated repair process: it can either choose the corresponding homolog (two chromatids available) or the fully identical sister chromatid. As the sister chromatids in G₂ are still intimately connected by cohesion, whereas the homologous chromosomes are usually further apart, the templated repair in G₂ is strongly biased towards the sister chromatid. If a meiotic cell would naively apply the same mechanism by default, repairing the Spo11-induced DSBs off the sister as a template, this would have no effect genetically at all; so this is not a common option. Crossover-type exchange events require productive interaction with the homolog instead. Somehow, the potential recombination with the sister chromatid has to be actively suppressed, in spite of its close proximity, but how this happens is still under active investigation.

Some circumstantial evidence exists in *S. pombe* that the Spo11 equivalent is loaded onto DNA together with the establishment of sister-chromatid cohesion (G. Cromie and G.R. Smith, this SERIES). In budding yeast, this cohesion is established during S phase (Uhlmann and Nasmyth 1998; Lengronne et al. 2006). These cues may be the most relevant for grasping the molecular basis for partner choice bias in meiotic crossing-over. Also in budding yeast, the screening for partner choice mutants has pointed at several relevant candidates (Thompson and Stahl 1999). Among other functions, a meiosis-specific checkpoint kinase (Mek1) plays a critical role in these controls (Perez-Hidalgo et al. 2003; Niu et al. 2007). Based on the close juxtaposition between meiotic sister chromatids, an integrated model has been proposed assuming the coordinated assembly of a regional “barrier to sister chromatid repair” (BSCR) wherever a functional Spo11 complex has been loaded on to (and/or activated on) the other chromatid (Niu et al. 2005).

5

Searching for Homology

Finding the needle in a haystack

Crossing-over during meiosis is directed at interacting chromosome pairs of homologs; it is “homologous recombination” (HR) in a nutshell. Yet, to engage in HR productively at any given site, sufficient “homology” at the

¹³ Relative to DNA replication (synthesis) at S phase, the interphase between mitotic divisions is described by two gaps, G₁ before replication and G₂ after.

DNA level must first be ascertained beyond a predetermined threshold, discriminating against randomly occurring shorter stretches of DNA sequence similarity within nonhomologous surroundings. Ideally, DNA homology is defined as residual sequence identity by descent, rather than the coincidental similarity arising from stochastic variation. The enzymes performing HR, however, can only go one way or the other by directly comparing two sequences at a time, with rather few independent cues as to their likelihood of sharing a common ancestry. How can such enzymes quickly and reproducibly fulfill this role?

The archetypal enzyme for assessing the degree of homology between a 3' end of ssDNA and potential double-stranded target sequences is the RecA protein of *Escherichia coli* (C. Prévost, this SERIES). This protein is involved in DSB repair¹⁴; RecA orthologs exist in Archaea (RadaA) and in eukaryotes (Rad51). In general, eukaryotes carry a meiosis-specific paralog (Dmc1) and may have additional Rad51 paralogs as well. These important members of a DNA-dependent ATPase family have in common that they can assemble as helical filaments on ssDNA (~1 kb or even longer), which in turn can intertwine with dsDNA of any sequence.¹⁵ Due to the rigid scaffolding provided by the surrounding protein filament, the target duplex DNA is stretched and partially unwound, which greatly reduces the base pair stacking forces. It also allows base pairing to switch coordinately between the strands – AT pairs first (reversibly) and GC pairs later on, when a high number of matching AT pairs indicates a sufficient degree of sequence homology along the so-called presynaptic filament (Folta-Stogniew et al. 2004). Most current models assume that the exchange of base pairing during the partner switch occurs by a sliding movement of individual bases, within the plane of their aromatic rings (C. Prévost, this SERIES). An alternative model preserves the remarkable symmetry of a quasi-quadruplex configuration¹⁶, if base exchange occurs by flipping 180° about the stationary glycosidic bonds (Egel 2007).

A major problem, in fact, is one of great numbers. For every matching target of a long identical sequence there exist ever so many others that do not fit, and the prospective RecA filament on ssDNA cannot judge beforehand whether or not a random encounter with a potential duplex target happens to be a proper match. To find out, the searching filament actually has to fully intertwine in register with the duplex sequence and start the base pair exchange reaction over an extended length. Every futile encounter with a heterologous sequence has to be reversed completely before the next attempt can be

¹⁴ Double-strand breaks arising from environmental damage or the collapse of stalling replication forks.

¹⁵ In general, just three intertwining strands of DNA are accommodated inside the helical RecA filament. There is room, however, for a fourth strand to participate in the exchange reaction, without distorting the protein filament (Mazin and Kowalczykowski 1999).

¹⁶ In cross-section, the three participating strands occupy three corners of a square.

initiated at a new site. It should be helpful, of course, if the filament could somehow “remember” and avoid the unproductive encounters tried before, but RecA alone is not capable of unidirectional scanning along a duplex target for a suitable start (Adzuma 1998). Additional proteins may serve as processivity factors to raise the efficiency of RecA-type filaments in this regard.

In eukaryotes, a series of other proteins associate with Rad51 and/or Dmc1 filaments, and all of them are required for full recombinase activity. Thus, one of them (“RadX”) might act as a processivity factor. In brief, the RadX-modified Rad51 filament could work as follows: Instead of wasting valuable time with probing heterologous sites at any length, the 3′ end could sweep along a narrow sliding window until it finds a perfect match. The sudden drag arising from many flipped base pairs could then allow the sliding window to be widened for probing further down the line. In the rare case of having found the perfectly homologous target, this should launch an avalanche of instantaneous fit, comprising essentially all the base pairs in a row. When such a perfect match has been accomplished, the RadX–Rad51 filament is disassembled and the stretch of heteroduplex DNA is passed on to other protein complexes for further processing.

A candidate of particular interest among the recombinase-associated factors is Rad52. Among the corresponding mutants, lack of Rad52 has the strongest effect, working relatively early in presynaptic filament formation (New et al. 1998), and the entire series is termed the *RAD52* epistasis group of genes. Also, both yeast and human *RAD52* proteins form heptameric ring structures, which bind preferentially to the ends of ssDNA (Shinohara et al. 1998; Parsons et al. 2000); this should be the most suitable site for a processivity factor.

6 Homolog Pairing and Synapsis

Keep in touch!

Trying to understand the initial strand exchange reaction between homologous DNA molecules may appear intricate enough; the choreography and orchestration of meiotic crossovers at the levels of chromatin and entire chromosomes is yet a different matter. In general, crossovers are not placed randomly along the chromosomes, but the molecular mechanisms behind the biased choice are still not fully understood. Both positive and negative factors influence the bias.

As each bivalent of homologs should at least have one chiasma, initial factors tend to raise the chances of getting one. For instance, physical tethers can connect the pairs of homologs after an incidental first encounter, which thereafter reduces the risk of drifting apart, thus increasing the chances for

further productive encounters elsewhere on the same bivalent. Several successive steps can be distinguished, such as initial recognition, presynaptic alignment, and synapsis (S. Mehrotra, R.S. Hawley and K.S. McKim, this BOOK; N. Hunter, this SERIES; Tesse et al. 2003; Lui et al. 2006). Only the initial recognition occurs independently of Spo11 activity (creating DSBs along the chromosomal DNA). The approximate alignment (also referred to as the pairing stage) and full synapsis by the widely conserved synaptonemal complex (SC) often require DSBs and DNA-dependent interactions. The initiation of pairing and/or synapsis on individual chromosomes can vary greatly between different organisms and even between genders of the same species. In human males, for example, initiation of pairing and synapsis invariably starts close to the telomeres (Brown et al. 2005). This correlates well with male-specific differences in the genetic map, as well as the distribution of chiasmata. Both crossing-over and chiasmata are preferentially observed close to the telomeres in male meiosis, in contrast with a more interstitial distribution during oogenesis in females (C. May, T. Slingsby and A.J. Jeffreys, this BOOK). This male-specific favoring of subterminal chiasmata may be related to the pseudoautosomal pairing regions (only 2.7 and 0.33 Mb) at either end of the otherwise nonhomologous X and Y chromosomes. The obligate chiasma observed in the major one of these makes this the “hottest” hotspot region in the entire human genome.¹⁷ Although the independent initiation of synapsis at multiple interstitial sites has not yet been demonstrated in human oogenesis, it has been shown for numerous species with more readily accessible meiotic material (von Wettstein et al. 1984).

The occurrence of recombination-independent pairing sites is prominent in the achiasmatic meiosis of *Drosophila* males, where these contacts alone can stabilize the bivalents until metaphase I (Sect. 1.1). Preferential pairing sites of lesser stringency are also known for *Drosophila* females (S. Mehrotra, R.S. Hawley and K.S. McKim, this BOOK). In a yeast, too, pericentromeric heterochromatin association can act as a meiotic pairing site (Davis and Smith 2003). Also in fission yeast, a particularly striking example is at the *sme2* locus, which encodes a nontranscribed RNA required for the progression through meiosis. Notably, the RNA-binding inducer protein of meiosis, Mei2, aggregates specifically as a dot structure at the *sme2* locus (Shimada et al. 2003). The functional *sme2* locus has since been shown to act as a strong recombination-independent pairing site (D.Q. Ding and Y. Hiraoka, personal communication). This demonstrates that a particular RNA can organize a nucleation center for homolog pairing at the site of its transcription. At a different level, the association of meiotic telomeres to the nuclear envelope and their preferential clustering in the widely conserved bouquet arrangement (Sect. 6) can likewise increase the chances of homologous loci approaching one another in meiotic prophase.

¹⁷ The minor region of 0.33 Mb only contributes with one chiasma per 25 meioses.

The most conspicuous identifier of meiosis at the ultrastructural level is homolog synapsis as detected by the presence of SCs.¹⁸ With rather few exceptions, the uniform SC structure is observed in every branch of eukaryotes, and some of its components belong to the conserved core set of meiotic proteins (Villeneuve and Hillers 2001). It is assembled processively from few starting points by connecting the axial cores of homologous chromosomes with fibrous proteins across the central space. In the lateral elements of the SC, the core structures of sister chromatids are still intimately united (J.A. Suja and J.S. Rufas, this BOOK), and the individual chromatid cores are only separated after SC structures have been disassembled at the diplotene stage.

As to the actual role of the SC in mainstream meiosis, the predominant view has long been that its main function should be crucial in facilitating crossing-over by keeping the homologs in register. The cause-effect relationship, however, no longer appears to be so simple, and not all organisms behave the same in this regard. While *Drosophila* indeed requires the SC to initiate the meiosis-specific DSBs that precede meiotic crossing-over (S. Mehrotra, R.S. Hawley and K.S. McKim, this BOOK), this dependency appears to be reversed in yeast (Henderson and Keeney 2004; S. Keeney, this BOOK). One way or the other, the transformation of selected DSBs into chiasmata, including the substantial restructuring of chromatid cores with these events, appears to occur in close association with the synaptonemal complex. On the other hand, the zipper-like assembly of SCs can be quite independent of local DNA homology, which is especially evident in structural heterozygotes for chromosomal rearrangements, where normal-looking SC structures can be observed between heterologous segments (von Wettstein et al. 1984).

SC formation and recombination can also be uncoupled in other exceptional cases. In the achiasmatic meiosis of *Bombyx mori* females, SC structures are modified and stabilized until metaphase/anaphase I, when compacted chunks of central-component material are liberated as so-called elimination chromatin (Rasmussen 1977a). Conversely, in the asynaptic meiosis of fission yeast, central SC components do not form at all, in spite of high levels of crossovers per chromosome in this organism (G. Cromie and G.R. Smith, this SERIES).

If it is not crossing-over per se, could there be other important SC functions to warrant the widespread evolutionary conservation of this meiotic structure? There is, in fact, a substantial risk of physical interlocking between two or more nonhomologous bivalents. This hazard occurs if synapsis is initiated at multiple sites in the same bivalent and another chromosome arm is trapped in the middle, in turn forming an entrapped bivalent with a fourth

¹⁸ The classical stages from light microscopy can be redefined with respect to SC formation: Leptotene, axial cores present, no SC; Zygotene, partial presence of SC, with separated axial cores in between; Pachytene, full synapsis with contiguous SCs in all the bivalents; Diplotene, disassembly of SCs, separation of lateral elements, as followed by separation of chromatid cores.

chromosome. Such interlocks can indeed be relatively frequent during zygotene, but they have usually disappeared at pachytene (Holm et al. 1982). Thus, they can be repaired efficiently. As the entrapped chromosome arms consist of two tightly connected sister chromatids, both these chromatids have to be severed simultaneously and correctly sealed thereafter. A suitable enzyme for such repair is topoisomerase II, which is abundant as a structural component of chromatid cores (J.A. Suja and J.S. Rufas, this BOOK). It is not unreasonable to assume that the resealing of “double DSBs” upon interlock resolution is greatly facilitated by SC formation on both sides, since the unbroken lateral component of the homolog should automatically attract the broken ends and bring them into close proximity. Notably, the resolution of synaptic interlocks is significantly impaired by SC anomalies, as observed in hybrid cattle bearing partly heterologous chromosomes (Dollin et al. 1991).

7

Crossover Interference

To count or not to count?

Crossover interference is a mathematically defined descriptive term related to the fact that the absolute numbers of crossovers (chiasmata) in general are clustered more closely around a given mean value than expected for a random distribution (Poisson). As the observed mean value often lies closely above one chiasma per bivalent for the shortest chromosomes, or about two to three for longer ones, the Poisson formula would, in fact, predict an unacceptably high risk of receiving no chiasma at all.¹⁹ Yet, this zero class is largely suppressed in wild-type specimens. Correspondingly, the number of multiple crossovers, especially within shorter intervals, is likewise reduced below the level expected for a random distribution. Increasing the likelihood of the first chiasma on a bivalent is often referred to as the “obligate crossover”. Conversely, the reduction of multiple events below the expected average is termed crossover interference.

Although crossover interference has been known for about 90 years, a unifying mechanism for this complex issue has not yet been ascertained. There appear to be two mechanisms leading to meiotic crossing-over, one that is associated with interference and another one which is not (G.H. Jones and F.C.H. Franklin, this BOOK; J.E. Haber, this SERIES). Both pathways are overlapping and more or less redundant, and their relative importance can vary greatly between organisms.²⁰ They have only been revealed by painstaking

¹⁹ As mentioned before, the accidental lack of chiasmata would bear a high risk of meiotic nondisjunction.

²⁰ At the extremes, all crossovers in *S. pombe* are without interference, while in *Caenorhabditis elegans* they all do show interference.

analyses of various mutants, where one or the other pathway is affected differentially.

It has long been known from genetic experiments that most gene conversion events that are not associated with a crossover do not cause interference on other crossovers in the vicinity. More recent mutant analyses in yeast ascribe the interference-prone pathway to the action of the Dmc1 recombinase and the proteins interacting with this meiosis-specific RecA homolog (J.E. Haber, this SERIES). The major recombinase of general DSB repair, Rad51, does not cause interference with additional crossover events, although it can efficiently drive meiotic crossing-over in the absence of Dmc1. Other proteins interacting with Dmc1 in the interference-prone crossover pathway include Rdh54 (the presumptive processivity factor; Sect. 5), as well as the Hop2–Mnd1 complex, which appears to link this pathway to the proper establishment of homolog synapsis. The synaptonemal complex, therefore, may well serve a structural role as a scaffold to mediate the interfering influence between closely spaced potential crossover initiation sites.²¹ Furthermore, the two crossover pathways in yeast differentially depend on different protein complexes related to mismatch repair functions.²² The interference-prone pathway requires Msh4–Msh5 and, to a lesser extent, Mlh1–Mlh3, whereas the non-interference pathway requires Mus81–Mms4 (Argueso et al. 2004).

In a complementary cytological approach, meiotic interference has also been observed for the spatial distribution of Mlh1 protein, which forms distinct foci in meiotic prophase, correlating with recombination nodules (T. Ashley, this BOOK). From mutant studies in the mouse it has been claimed that this interference can be uncoupled from full synapsis (de Boer et al. 2007). Also in the mouse, it had been shown before that meiotic cohesin complex proteins can form fibrillar core-like structures (and attract recombination proteins, such as Dmc1) in the absence of axial-element proteins that normally contribute to SC formation (Pelttari et al. 2001). In tomato as well, Mlh1 foci show strong spatial interference in a subset of recombination nodules (Lhuissier et al. 2007). Late recombination nodules are considered to represent subsequent crossover sites, among which the Mlh-positive nodules may represent the “obligate” chiasma sites.

These and related data show that modern studies on crossover interference have moved this once esoteric field from a peripheral digression in genetics textbooks to the forefront of molecular biology. It is from analyses along these lines that the most significant progress in our understanding of the recombinational mechanisms in meiosis can be expected.

²¹ Notably, the crossover-proficient, yet asynaptic, meiosis of fission yeast does not show crossover interference (G. Cromie and G.R. Smith, this SERIES). Also, the usual discrimination against meiotic sister chromatid exchange is not observed in this organism.

²² While the functions of these proteins in eukaryotes are less clear, the respective sequences are partly homologous to established mismatch repair genes in *E. coli*.

8 Telomere Clustering

A loopy bouquet

In many organisms studied, the choreography of meiotic chromosome movements opens with a graceful dance. At early prophase, all the chromosomes attach to the nuclear envelope with both their telomeres, which in turn are gathered in a cluster at one side of the nucleus (Scherthan 2007). The bulk of every chromosome then forms an extended loop through the inner lumen of the nucleus. This so-called bouquet arrangement usually coincides with the conventional stages of leptotene and zygotene, i.e., the beginning of synapsis (Fig. 1).

In the numerous examples where chiasmata preferentially occur close to the telomeres (including the human male, Sect. 6), the initiation of pairing may be greatly facilitated by the clustering of telomeres in the bouquet arrangement. Similar to what has been described in Sect. 6, the independent initiation of synapsis at both ends of a chromosome can lead to the interlocking between nonhomologous bivalents, even in the achiasmatic *Bombyx* females (Rasmussen 1977b). To further correlate the bouquet arrangement with homologous synapsis, both processes can be affected by the same mutant in maize (Golubovskaya et al. 2002).

In most organisms, the clustering of telomeres is driven by actin filaments. A notable exception, again, is observed in the asynaptic meiosis of fission yeast where cytoplasmic microtubules are involved and the bouquet arrangement is modified considerably (D.Q. Ding and Y. Hiraoka, this SERIES). As a direct continuation of the nuclear movements preceding karyogamy, the zygotic fusion nucleus is repeatedly moved back and forth throughout the entire phase of the bouquet arrangement in this organism.²³ The dynamic strokes are driven by a bundle of cytoplasmic microtubules, pulling the spindle pole body²⁴, together with the drawn-out nucleus, over the entire length of the zygotic cell. At this stage the telomeres are attached to the SPB inside, transiently replacing the centromeres at that center of dynamic activity. Functionally, the “horsetail” movement replaces the lacking synapsis, by transiently aligning the stretched-out homolog loops to some extent.²⁵

Conceptually, the telomere clustering observed during the bouquet stage at meiosis represents an alternative mode of moving chromosomes, as compared to the conventional mitotic spindle mechanism. Recently the interest-

²³ Similar nuclear movements are also observed without karyogamy in (artificially selected) diploid cells of *S. pombe* induced for meiosis.

²⁴ The spindle pole body (or SPB) is integrated in the nuclear envelope and represents the fungal equivalent of a centrosome in animals.

²⁵ Haploid *S. pombe* only has three chromosomes, which differ in length, allowing loops of equal length (the homologs) to move coordinately in approximate alignment.

ing notion has been presented that centromeres may have derived from the evolutionarily older telomeres (Villasante et al. 2007). According to this view, the bouquet arrangement may indeed represent a very ancient aspect of eukaryotic evolution (Sect. 10), which among contemporary organisms only has survived at the meiotic stage of the life cycle.

9

Meiotic Spindle Dynamics

Pushes and pulls

After desynapsis at diplotene has revealed chiasmata, the visible results of meiotic crossing-over, it is the duty of the spindle apparatus to separate half-bivalents²⁶ in meiosis I (Fig. 1). Both meiotic and mitotic spindles have much in common. They represent one of the most intricate, self-organizing protein machines of the eukaryotic cell. Basically they consist of microtubules (MTs), various motor proteins and other MT-interacting proteins, as well as anchor facilities at the various client targets to be agitated. When a division spindle is fully operational at metaphase and anaphase, it comprises two poles and numerous MT bundles in between. At large, there are two ways of building up this overall organization, a *top-down* and a *bottom-up* approach to self-assembly, both giving similar outcomes.

Predominantly, as prevailing in mainstream mitosis and also in metazoan spermatogenesis, the division and separation of preexisting centrosomal structures play a leading role (*top-down*). Before oogenesis, however, centrosomal proteins often disappear and the first meiotic spindle is built up in a decentralized manner (*bottom-up*). Functional centrosomes, which again are required for the somatic cell divisions during embryogenesis, are in turn provided by the fertilizing sperm. Together with other measures, this functional asymmetry may prevent unfertilized eggs from developing autonomously by parthenogenesis. More recently it has become apparent that centrosome-containing animal cells, in fact, are assisted by the secondary pathway as well (Rieder 2005); the centrosome-less plant cells, on the other hand, form all their spindles by the second route.²⁷

To point out the various aspects of MT-based dynamics and chromosome motility, the basic constituents are presented in brief, and the most important interactions are discussed. The hollow MT structures are composed of stacked α/β tubulin dimers, with a directional polarity. They are nucle-

²⁶ Each half-bivalent consists of two partly recombined chromatids connected to one set of parental sister-centromeres.

²⁷ In addition, fungal cells have a “closed mitosis”, where the nuclear envelope remains intact during nuclear divisions. Their spindle pole body (SPB), embedded in the nuclear envelope, is functionally equivalent to metazoan centrosomes.

ated at their stable minus ends, as mediated by special complexes containing γ -tubulin (Raynaud-Messina and Merdes 2007), and they either grow or depolymerize at their dynamic plus ends. The disassembly, in particular, can be quite rapid ("MT catastrophe"); it generally occurs by splaying apart of the tubulin dimer protofilaments, when these bend out from the straight arrangement in the MT lattice to a curved configuration in the terminal whiskers (McNally 1999). The shift from the growing mode to rapid disassembly and vice versa is modulated by a wide range of accessory proteins. Various motor proteins, such as the dynein or kinesin families, can track on MTs to their plus or minus ends, thus pulling specific cargoes in the same direction. Side-by-side arrangements and sliding in bundles are very common, especially between antiparallel MTs, as mediated by crosslinking proteins (Janson et al. 2007).

The centrosome-directed spindle consists of antiparallel pole-to-pole fibers, which overlap in the central zone and push the poles apart. Additional MTs are continuously nucleated at the centrosomes; their dynamic plus ends can be captured by the kinetochores, where poleward pulling force is generated, either by anchored motor proteins (Nicklas 1997; Mimori-Kiyosue and Tsukita 2003) or an alternative mechanism (see below). If the sister kinetochores of mitotic chromosomes (or at meiosis II) are thus attached to opposite poles, the opposing pulling forces cancel out in the stable metaphase arrangement. The resulting tension is sensed across the centromere, which successively deactivates a checkpoint control mechanism, in turn allowing sister centromere cohesion to be lifted when the last chromosome has been attached to both spindle poles (Musacchio and Salmon 2007).

Actually, the kinetochores are not entirely passive before encountering a centrosome-anchored MT merely by chance. Additional MTs can, in fact, be nucleated at the kinetochores themselves (K-fibers, Maiato et al. 2004), which requires the multiprotein "chromosomal passenger complex" (Sampath et al. 2004; Vader et al. 2006), as well as the Nup107–160 nucleoporin complex (Orjalo et al. 2006). By directly interacting with the centrosomal spindle, these K-fibers can greatly accelerate and stabilize the bipolar attachment of all the chromosomes or bivalents in mitosis or meiosis, respectively. Two minus end-directed motors, dynein and Ncd, have been implicated in focusing the K-fibers and transporting them towards the poles (Goshima et al. 2005).

This pathway is still operative in centrosome-lacking oocytes, as well as in plant cells. There, the nascent spindles are generated directly at the chromosomes, initially from smaller bundles of antiparallel MTs at individual chromosomes, which in turn cooperate in a preferential direction and finally coalesce to a bipolar spindle arrangement. Also, the meiotic spindle checkpoint appears to be active in oocytes (Wang and Sun 2006), although its reduced efficiency may be responsible for the high risk of nondisjunction observed for human oogenesis (H. Kokotas, M. Grigoriadou, and M.B. Petersen, this BOOK).

Motor proteins at the kinetochores have long been held responsible for the generation of poleward pulling force at metaphase and anaphase (Nicklas 1997; Rieder and Salmon 1998). A traction fiber model, assuming motor activity all along the K-fibers, has also been considered (Pickett-Heaps et al. 1997). More recently, a motor-independent alternative has been suggested, where latent energy stored in the spindle fibers is reclaimed from MT depolymerization. The fraying whiskers at depolymerizing MT ends can, in fact, exert sufficient driving force to move a microbead under the microscope (Grishchuk et al. 2005). To harvest this energy efficiently at the kinetochore, the ten-protein Dam1 complex discovered at yeast kinetochores appears very appropriate. In vitro, this complex oligomerizes as a loosely fitting ring around MTs (Westermann et al. 2006). Such a tethered ring should be suitable to transform the recoiling force of all the radially outward bending MT protofilaments at the depolymerizing end into linear motion towards the pole. This mechanism would, in fact, be readily compatible with earlier observations on permeabilized (thus energy-depleted) mitotic cells that anaphase movements could still be powered by energy stored in the spindle (Spruck and Pickett-Heaps 1987).

There is a particular aspect of the familiar metaphase arrangement that still awaits a mechanistic explanation. Evidently, the opposing forces cancel out if all the mitotic chromosomes, or bivalents at meiosis I, are assembled in an equatorial ring around the spindle. The equidistant symmetry to the poles implies that the forces acting on the kinetochores should vary in proportion to the momentary length of the chromosomal spindle fibers. Only then could many asymmetric placements (expected during early metaphase) be corrected coordinately: by always shortening the longer connection and extending the shorter one. In the traction fiber model, of course, the number of motor proteins per traction fiber could be proportional to fiber length. If essentially all the force, however, is generated at the kinetochores, some critical cofactors should be loaded in proportion to fiber length and subsequently be delivered at the kinetochores by directional movement. These cofactors remain to be characterized.

To conclude this section on spindle dynamics, a peculiar meiosis-specific phenomenon appears worth mentioning. This concerns the dynamic behavior of univalents at prometaphase of meiosis I. As their sister kinetochores are still fused as a functional unit, univalents should only connect to one pole or the other, but not to both poles simultaneously. Certain insects with X0 sex chromosomes, such as grasshoppers, show univalent X segregation in each meiosis during spermatogenesis. In such material, when all the autosomal bivalents assemble in the equatorial metaphase arrangement, the X univalents make several movements from one pole to the other. The movement is quite uniform throughout the entire path, but ceases close to the pole. Then it takes a variable time for the kinetochore to reorient and resume the uniform movement towards the opposite pole (Nicklas 1961). Autosomal univalents have

been studied in crane fly spermatogenesis, essentially giving the same result (Steffen 1986). The latter material was also inspected by electron microscopy. During the intermittent stage of reorientation, the kinetochore was found in direct contact with a laterally associated MT bundle instead of making a direct pole connection.

The regular occurrence of kinetochore reorientation and reconnection to the opposite spindle pole is highly relevant for bivalents as well. It is not uncommon that the homologous centromeres in a bivalent both move to the same pole initially. Frequent malorientation can also be provoked experimentally by cold treatment to study recovery from such anomalies (Henderson et al. 1970; Janicke and LaFountain J.R. Jr 1986). Thereby it has been verified that efficient reorientation of initially maloriented bivalents does indeed occur. This is an important safeguard against meiotic nondisjunction, and it is the task of the tension-responsive spindle checkpoint to provide the necessary time for bipolar spindle attachment to be achieved.

10

Evolutionary Remarks

Rather little may make sense in biology, were it not for Darwinian evolution

Brooding on evolutionary issues usually reduces to questions about “*the chicken or the egg*”. Often such questions cannot be answered from within a narrow frame, but widening the scope may lead to significant insights simply by twisting the original question in a novel way. Were there chickens before eggs? The answer is *No, for all we know*. Yet, were there eggs before chickens? *Yes, there certainly were*, only most of those were definitely not chicken eggs. That is what evolution is about.

In the context of these two volumes on *Recombination and Meiosis*, three evolutionary topics have been selected for a general discussion, putting the molecular details of recombinational mechanisms in a wider perspective:

- (i) In metazoans, such as ourselves, meiosis is linked to the magic singularity of our life cycle when differentiation into many complex body tissues no longer matters and the essence of life is reduced back down to the level of two single cells, which subsequently fuse as a single zygote, so as to start another composite being (almost) from scratch. Not all the cells in our bodies are still capable of giving rise to germ cells. The few that can do so are known as the germline. Dirk Lankenau (this SERIES) follows the germline concept from August Weismann’s prescient propagation of the *germ-plasm’s continuity* (Weismann 1892, 1893) to the present day. In particular, he identifies Charles Darwin’s and August Weismann’s cognitive insights into the existence and power of selectional hierarchies at multiple levels – each representing its own biological entity – and dis-

cusses the driving factors for the maintenance of sex in metazoans from the population genetics point of view.

- (ii) Sexual propagation, including meiosis, abounds among all the major branches of multicellular eukaryotes. Accordingly, meiosis has been considered a *preadaptation*²⁸ for the evolution of multicellularity (Maynard Smith and Szathmari 1995). Nevertheless, meiosis has been lost repeatedly in various lineages, some of which have lasted several million years. Isa Schön reviews the absence of meiosis in three major examples of such “*ancient asexual scandals*” (I. Schön, D.K. Lamatsch and K. Martens, this SERIES). It now appears that ameiotic recombination can be quite efficient in keeping deleterious mutations at bay, certainly more so than previously anticipated.
- (iii) The ultimate chicken-and-egg problem begs the question: *Were there eukaryotes before meiosis?* In their *coevolution hypothesis* for the origin of meiosis, Richard Egel and David Penny (this SERIES) are inclined to state, *probably not*. The recognition of a universally conserved core set of meiotic proteins (Villeneuve and Hillers 2001; Ramesh et al. 2005) has pushed back the origin of meiotic sex below the latest common ancestor for all the eukaryotic lineages still living now. At this ultimate level below the latest branching point of interest, there is little to compare for further resolution, and quantitative reasoning by statistical arguments loses its decisive role. Instead, a critical reevaluation of basic assumptions about early biotic evolution may shed new light on the putative origin of meiosis as well.

Meiosis is a very complex system, coordinated by many genes and corresponding protein functions. How could such a complex network ever be invented? In fact, a comparable system has never been reinvented another time in any lineage that had lost meiosis for good. Instead, the alternative notion has been put forward that mitosis and meiosis could have evolved together from the very start, as alternating programs of genome maintenance and propagation that were optimized in parallel by regularly alternating environmental conditions (R. Egel and D. Penny, this SERIES). As for the origin and evolution of complex morphological novelties, a general theory has been developed (Budd 2006), relying on *functional continuity*, *redundancy*, and *preadaptation*. In being a complex trait as well, meiosis has likely been facilitated or constrained by similar evolutionary factors.

For the purpose of this introductory synopsis, I briefly mention three putatively ancient traits that had preadaptive value for being reutilized in the meiotic program:

- (i) Recombinational repair activities, including RecA-type recombinases, are present and important in all three domains of cellular life. Their prin-

²⁸ A preexisting morphological or functional trait is ascribed a preadaptive value if it can readily be reutilized in a different context.

cial role is in repair of DSBs, which can arise by environmental damage or from internal causes, such as stalling replication forks or reactive radicals produced during oxidative respiration. The entire pathway has been reutilized in meiotic crossing-over, in part with duplicated components, such as the meiosis-specific Dmc1 recombinase (Sect. 5). The “damage” needed to start this recombinational repair is provided by a specific component, mentioned next.

- (ii) The cut-and-paste activities of topoisomerases are needed by all organisms to release the torsional stress of replication and/or to resolve interlocking of topologically constrained loops of DNA. A particular topoisomerase of ancient (archaeal) origin (Spo11 ~ topoisomerase VI) has been reutilized in meiotic prophase to inflict damage on the DNA in a controlled and manageable way (Sect. 4). A gyrase-type topoisomerase of bacterial origin (topoisomerase II) has likely replaced the original function of topoisomerase VI in vegetative cells.
- (iii) The peculiar clustering of telomeres at the bouquet stage of meiotic prophase (Sect. 8) is here considered an ancient trait of moving eukaryotic chromosomes, preceding the development of centromeres and their attachment to the mitotic spindle (Villasante et al. 2007). For telomeres to have fulfilled a centromere-like function in protomitotic segregation, this would have required that both telomeres of each chromosome interacted specifically before the looping sister chromatids could disjoin in a coordinated way. Such telomere–telomere interaction could have been the primary function of site-specific pairing factors and/or synaptic filaments, which thus had preadaptive value for being adopted for meiotic synapsis as well. The original mitotic role, presumably, has since been superseded by the more efficient conventional spindle apparatus, and the bouquet stage has predominantly survived in meiotic prophase.²⁹

The putatively ancient origin of these key meiotic activities is more compatible with the meiotic program being about as old as mitosis, rather than a more recent *de novo* invention. Other meiosis-specific modifications, such as concerning sister chromatid cohesion, centromere protection in meiosis I, or cell cycle modulation of meiosis II, could readily have derived by duplication from general mitotic components.

Presumably, the coevolution of meiosis with mitosis allowed the early, unicellular eukaryotes to cope with a seasonally changing environment.³⁰ Under rapid-growth conditions, mitosis allowed the presumably haploid cells to multiply identically, with constant selection for household functions essential

²⁹ Another niche for survival of this putative “molecular fossil” could be the amitotic division of macronuclei in ciliates, which are peculiar by harboring many more and smaller telomere-containing genome fragments than the generative micronuclei (see Katz 2001), but the details of this division mechanism are still unknown.

³⁰ This has long been a classic argument for the alternation between sexual and asexual generations in facultatively asexual populations (see I. Schön, D.K. Lamatsch and K. Martens, this SERIES).

for the vegetative cell cycle. At the end of seasonal growth, however, zygote fusion and meiosis allowed the cells to reconstitute a full complement of alternatively needed functions that were only essential for long-term survival in the dormant state (R. Egel and D. Penny, this SERIES). Due to significantly higher mutation rates early on, such a genetic recovery program would have been more vital during the primordial phase of cellular evolution than it might be today.

Last, not least, yet at a different level, our current understanding of meiotic recombination at the molecular level has also undergone a dramatic phase of intellectual evolution. This *evolution of recombination models* has aptly been reviewed by Jim Haber, and is to open the accompanying second volume in this SERIES.

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Chromatid Cores in Meiotic Chromosome Structure and Segregation

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Abstract There is an extensive literature concerning the structure of mitotic chromosomes and the participation of proteinaceous axial structures, the chromatid cores, in their organization. However, the involvement of the chromatid cores in the structure and segregation of meiotic chromosomes is less known. This chapter reviews the occurrence and behaviour of the chromatid cores in condensed meiotic chromosomes, particularly in grasshopper species. We present current models that relate chromatid cores with the lateral elements of the synaptonemal complex, a tripartite structure present along synapsed homologues during the pachytene stage of prophase I. Additionally, we summarize recent data about the relationship between the chromatid cores, DNA topoisomerase II α , condensin and cohesin complexes.

Keywords Meiosis · Chromatid core · Synaptonemal complex · DNA topoisomerase II α · Condensin · Cohesin · Segregation

Abbreviations

AEs	Axial elements
LEs	Lateral elements of the synaptonemal complex
NORs	Nucleolus organizer regions
PSCs	Poly-synaptonemal complexes
SC	Synaptonemal complex
SMC	Structural maintenance of chromosome protein
Topo II	DNA topoisomerase II α

1

Introduction

During cell division, chromosomes condense during prophase, align at the metaphase plate and then segregate to opposite poles during anaphase. Chromosome condensation and segregation during mitosis and meiosis are two fascinating events that have focussed the attention of many researchers for more than a century. During meiosis two successive divisions take place without an intervening DNA replication step, resulting in the generation of

haploid germ cells. During meiotic prophase I homologues pair, synapse and recombine. At metaphase I, recombined homologues appear as bivalents that reach an accurate biorientation since sister kinetochores are intimately associated in each chromosome. In these bivalents, sister chromatids are closely connected at their arms and centromeres by cohesin complexes, and probably by DNA catenations (for review see Murray and Szostak 1985; Miyazaki and Orr-Weaver 1994; Paliulis and Nicklas 2003). At anaphase I, sister-chromatid arm cohesion is released, and consequently, the recombined homologues separate and segregate to opposite spindle poles, each one carrying two chromatids that are only associated at the centromere region. During the metaphase II/anaphase II transition, sister-chromatid centromere cohesion is lost allowing chromatids to segregate towards opposite poles (Fig. 1) (see chapter by Y. Watanabe in this volume).

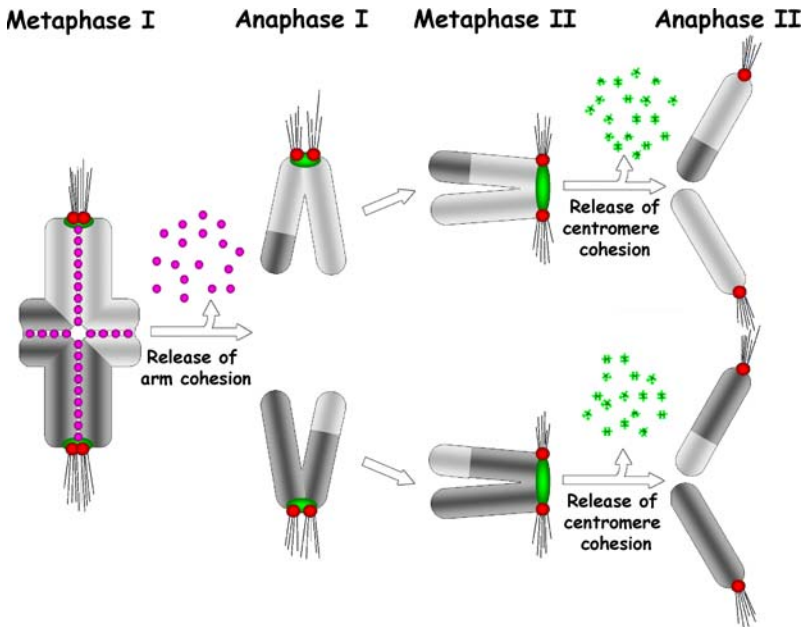


Fig. 1 Chromosome segregation during meiosis. One telocentric chromosome is depicted in *light grey* while its homologue is depicted in *dark grey*. Kinetochores are indicated as *small white balls* at centromeres. The metaphase I bivalent shows a single interstitial chiasma. In metaphase I, there are cohesin complexes at the arms (*light grey patches* at the interchromatid domain), and at centromeres (*dark grey oval*) below the closely associated sister kinetochores. During the metaphase I/anaphase I transition, the cohesin complexes at the interchromatid domain are released to allow the segregation of recombined homologues to opposite poles. The cohesin complexes at centromeres persist until metaphase II. During the metaphase II/anaphase II transition these complexes are released to permit the segregation of the chromatids. The composition of cohesin complexes may differ at the arms and centromeric regions

During the last two decades different investigators focused their research on the elucidation of the mitotic chromosome structure. However, the analysis of meiotic chromosome structure has received much less attention. There are extensive data in the literature describing the structure of prophase I chromosomes, particularly at pachytene, when a complete synapsis takes place between the homologues by means of the formation of the synaptonemal complex (SC). Nevertheless, less is known concerning the post-pachytene chromosomal structure.

In this chapter we first briefly summarize what it is currently known about the structure of mitotic chromosomes with particular emphasis on the chromatid cores as axial structures. Then, we introduce the organization of meiotic chromosomes. In this sense, we present the structure of SCs and their components in prophase I. Posteriorly, we describe what is actually known about the occurrence and behaviour of silver-stained chromatid cores in fully condensed grasshopper bivalents and univalents during both meiotic divisions. Finally, we present current opinions on the relationship between the chromatid cores and the SC lateral elements, DNA topoisomerase II α , and the condensin and cohesin complexes.

2

Mitotic Chromosome Structure

Mitotic chromosomes, particularly in metaphase, represent the highest level of packaging that chromatin may reach during the somatic cell cycle. In metaphase chromosomes, the DNA is compacted approximately 10 000-fold, while the interaction of DNA with histones results in a 30 nm diameter chromatin fibre with about 40-fold packaging of the DNA. During the last 60 years different models have been proposed to explain the 250-fold further compaction of these 30 nm chromatin fibres that shape metaphase chromosomes (Adolph 1988; Paulson 1988; Rattner 1988; Stack and Anderson 2001; Strukov et al. 2003; Swedlow and Hirano 2003; Gassmann et al. 2004; Kireeva et al. 2004). The “folded-fibre model” suggested a random folding of chromatin fibres in metaphase chromosomes (DuPraw 1965), while the “helical coiling models” support the hierarchical helical coiling of the 30 nm fibre into increasingly larger structures (Sedat and Manuelidis 1978; Belmont et al. 1987). However, the most widely accepted model of mitotic chromosome structure, which is featured in nearly all textbooks, is the “radial loop/scaffold model” (Earnshaw 1988; Paulson 1988). This model emerged from the observation of spreads of histone-depleted mitotic chromosomes by electron microscopy (EM) (Paulson and Laemmli 1977). In its simplest version this model proposes that the 30 nm chromatin fibre is gathered into radial loops, each containing 50–100 kb of DNA, whose bases are anchored to a fibrous network of non-histone proteins, the so-called chromosome scaffold, which

retains the overall size and shape of metaphase chromosomes (Paulson and Laemmli 1977). Biochemical studies demonstrated that two proteins, Sc1 and Sc2, were the major constituents of the scaffold (Lewis and Laemmli 1982). Sc1 was identified as DNA topoisomerase II α (topo II) (Earnshaw et al. 1985; Gasser et al. 1986), an enzyme that has the potential to decatenate tangled DNA strands by passing one double helix through the other by creating transient protein-linked double-strand breaks (Roca 1995). Immunofluorescence and immunoelectron microscopy data indicated that topo II was present as a longitudinal axis inside each metaphase chromatid (Earnshaw et al. 1985; Earnshaw and Heck 1985; Gasser et al. 1986). To reconcile the radial loop/scaffold model with classical cytogenetic observations of coiled metaphase chromatids (Ohnuki 1968), a more elaborated version implying the helical coiling of a 200–300 nm chromatid fibre, and thus of the inner scaffold, was proposed (Rattner and Lin 1985; Rattner 1992). This modified model was supported by immunofluorescence studies using anti-topo II antibodies that showed that the scaffold was helically folded in each metaphase chromatid, and that sister chromatids had opposite helical handedness (Boy de la Tour and Laemmli 1988; Saitoh and Laemmli 1994).

The radial loop/scaffold model was further supported by the characterization of Sc2, the second-most abundant scaffold component. Two independent studies showed that Sc2 was a member of the structural maintenance of chromosome (SMC) proteins, a family of conserved ATPases, that colocalized with topo II at the chromosome scaffold (Hirano and Mitchison, 1994; Saitoh et al. 1994, 1995; Hirano 1995). Shortly afterwards, it was demonstrated that Sc2 corresponded to SMC2. SMC2 forms a heterodimer with SMC4, which in turn is associated with three other non-SMC proteins to form a protein complex required for chromosome condensation and segregation (Hirano et al. 1997), now termed condensin I. Further studies have shown that condensin complexes and topo II are interdependent for their association to chromosomes, and both appear as a longitudinal axis along each metaphase chromatid in different species (Coelho et al. 2003; Hudson et al. 2003; Maeshima and Laemmli 2003). Recently, a second condensin complex, condensin II, has been characterized (Ono et al. 2003). Interestingly, the condensin I and II complexes appear at different times during chromosome condensation, and at alternating subdomains along each metaphase chromatid (Ono et al. 2003, 2004; for recent reviews see Swedlow and Hirano 2003; Losada and Hirano 2005; Nasmyth and Haering 2005).

2.1

Chromatid Cores in Metaphase Mitotic Chromosomes

Shortly after the radial loop/scaffold model was proposed (Paulson and Laemmli 1977), two studies demonstrated that in mitotic chromosomes of different mammalian species silver staining revealed axial structures inside

metaphase chromatids (Howell and Hsu 1979; Satya-Prakash et al. 1980). These so-called “chromatid cores” are observed at the inner regions of metaphase chromatids as lines extending throughout their length (Fig. 2). It was proposed that these silver-stained chromatid cores are related to the chromosome scaffold observed on histone-depleted metaphase chromosomes (Earnshaw and Laemmli 1984). In some instances, the chromatid cores appeared helically coiled in metaphase chromosomes in mammals (Satya-Prakash et al. 1980; Haapala 1985), grasshoppers (Nokkala 1985) and plants (Stack 1991), as was shown posteriorly for the distribution of topo II and condensin I and II complexes by immunofluorescence (Swedlow and Hirano 2003). This indicated that chromatid cores and scaffolds represent the same structure. In this sense, it was suggested that the term chromosome scaffold should be used to describe a biochemical subfraction derived from chromosomes, and not a structural term at all (Earnshaw 1988). Consequently, it is preferable to employ the term chromatid core (or chromatid axis) for these axial chromosome structures to which the bases of radial chromatin loops are anchored.

Careful analysis showed that chromatid cores do not reach the chromatid ends since some chromatin is detected beyond the ends of these cores. Interestingly, the telomeric DNA repeats, as the ends of the chromatid cores, are

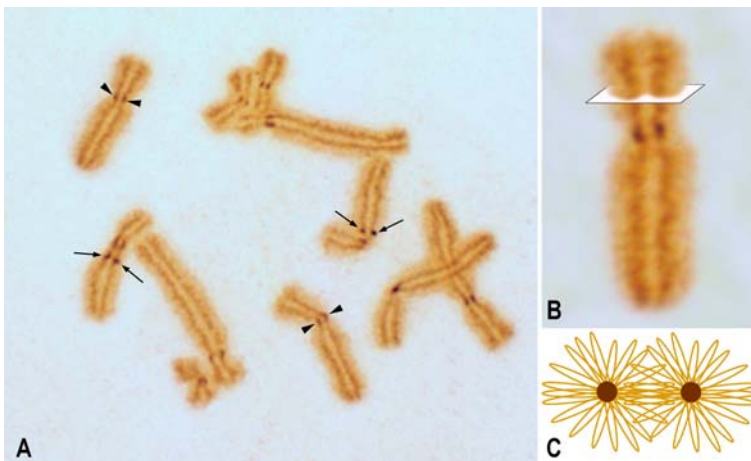


Fig. 2 Chromatid cores in mitotic chromosomes. **a** Chromosome spread of a metaphase rat kangaroo kidney epithelial (PtK₁) cell after silver staining. The 11 chromosomes present in this cell line show two silver-stained chromatid cores along their chromatids. In each chromosome, sister kinetochores (*arrowheads*), and the NORs (*arrows*) in the two X chromosomes, appear as core differentiations. **b** Selected submetacentric chromosome. Sister kinetochores and chromatid cores are evident. **c** Schematic drawing of an end-view of a cross-section through the chromosome in **b**. Each chromatid shows an internally located core from which chromatin radial loops emanate within a circle

not located at the chromatid ends, but there is some chromatin beyond them both in vertebrate and plant mitotic chromosomes, as observed by both light and electron microscopy (Meyne et al. 1990; Rawlins et al. 1991; Steinmüller et al. 1993). Additionally, a pair of round or slightly elongated structures representing sister kinetochores is also evident at the centromeric region as a differentiation of the chromatid cores (Fig. 2a,b) (Howell and Hsu 1979; Earnshaw and Laemmli 1984; Earnshaw et al. 1984; Fernández-Piqueras et al. 1984; Zhao et al. 1991). Moreover, in nucleolar chromosomes, the nucleolus organizer regions (NORs) are also preferentially stained, appearing as differentiations of the cores (Fig. 2a) (Howell and Hsu 1979).

3

Meiotic Chromosome Structure

3.1

Axial/Lateral Elements of the Synaptonemal Complex in Prophase I Chromosomes

The first recognized axial structures in meiotic chromosomes were the axial elements (AEs) and the lateral elements (LEs) of the synaptonemal complex (SC). Synapsis of homologous chromosomes during prophase I of meiosis involves the assembly of the SC, a meiosis-specific structure. The SC was described 50 years ago on longitudinal sections of animal pachytene spermatocytes observed by EM (Fawcett 1956; Moses 1956), and has been found in almost all sexually reproducing organisms. The SC is a proteinaceous ladder-like tripartite structure that is present along the entire length of the pairing surface between the homologues in a pachytene bivalent. The SC consists of two LEs, which can be revealed by silver staining, one per homologue, separated by a central space that contains a central element coupled to each LE by a series of transverse filaments (Fig. 3) (von Wettstein 1984; Heyting 1996; Zickler and Kleckner 1999; Page and Hawley 2004). During leptotene and zygotene the unsynapsed LEs are called AEs.

It is accepted that in meiotic chromosomes the chromatin fibres are organized in loops since it is known that the lampbrush chromosomes observed in amphibian oocytes during the diplotene/dictyotene stages of prophase I consist of loops radiating outward from a central axis (for references see Callan 1963; Rattner et al. 1980). Moreover, the analysis of spread pachytene spermatocytes and oocytes from different species shows that chromatin is organized as loops of different sizes, which are attached at their bases to the LEs (Moenes and Pearlman 1988; Heng et al. 1994, 1996). Deduced from longitudinal and cross-sections of pachytene bivalents from different species, it is clear that these LEs are peripherally located in relation to the chromatin loops from the sister chromatids of each homologue (Rattner et al. 1980; Von Wettstein 1984; Zickler and Kleckner 1999). We will refer to this kind of spatial arrange-

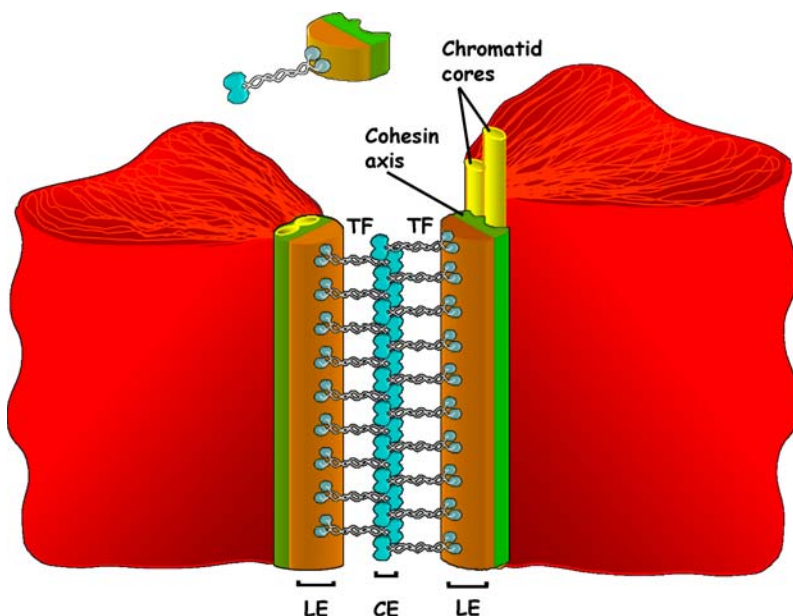


Fig. 3 Hypothetical model of SC structure. The chromatin loops from each sister chromatid are anchored at their bases to the chromatid cores, which are peripherally located with respect to each chromatid. A cohesin axis associates the sister cores, and acts as a framework for the assembly of lateral element (LE) proteins. The transverse filaments (TF) are depicted as homodimers with one end attached to LEs, and the other end conforming the central element (CE)

ment as “meiotic organization” or “meiotic chromosome structure”, which may be required for the essential events of pairing (alignment), synapsis and recombination between homologues.

The terms chromosome core, chromosome axis and homologue axis are frequently used to refer to a single axial structure per homologue during pachytene, i.e. the LE. To avoid confusion between the terms chromosome core and chromatid core, which are both revealed by silver staining, we will employ the term LE for the structure that appears along each homologue during pachytene, AE for this same structure during leptotene and unsynapsed regions during zygotene, and chromatid core (as defined previously) for the axial structure to which the bases of chromatin loops from a single chromatid are attached (Fig. 3).

3.2

Chromatid Cores in Metaphase I Bivalents

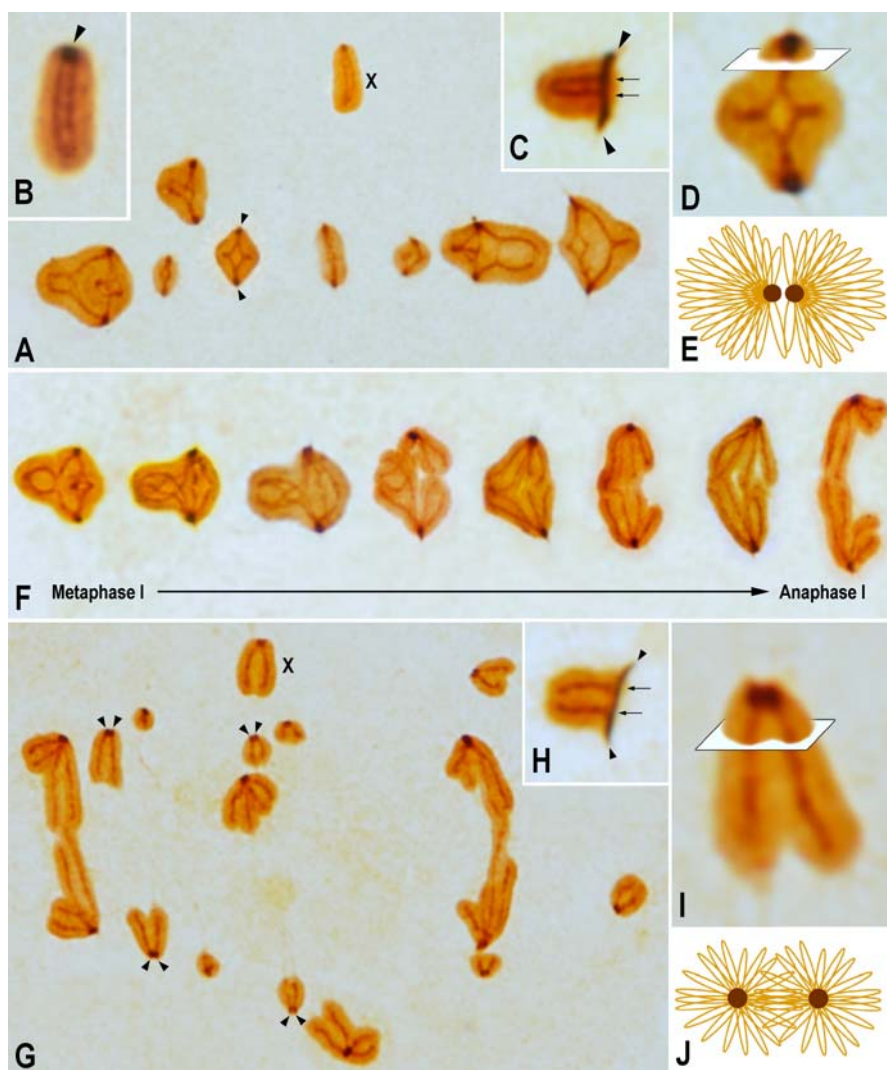
Silver staining has also been employed to reveal chromatid cores in condensed chromosomes during both meiotic divisions. First results were ob-

tained on grasshopper species (Rufas et al. 1982, 1983, 1987, 1988; Fernández-Piqueras et al. 1984; Sentís et al. 1984; Nokkala 1985; Santos et al. 1987), a classic material for the cytological analysis of meiosis since these species present a relatively low number of large chromosomes (Fig. 4) (Janssens 1924; John and Lewis 1965). Similar chromatid cores have also been demonstrated in plants (Stack 1991) and hemipterans (Nokkala and Nokkala 1985).

In grasshopper metaphase I bivalents, one silver-stained dot representing the two closely associated sister kinetochores is found at the centromere region of each homologue (Rufas et al. 1989, 1994) (Fig. 4a). Thus, each bivalent shows two “functional” kinetochores facing opposite poles. In addition, each chromosome shows two silver-stained cores, one per chromatid (Fig. 4a). These cores run along the chromatids from kinetochores to the distal telomeres although they do not reach the chromatid distal tips (Suja and Rufas 1994). The NORs, as occurs during mitosis, appear as chromatid core differentiations in nucleolar chromosomes (Giménez-Abián et al. 1989). If a bivalent composed of telocentric chromosomes and presenting a single in-

Fig. 4 Chromatid cores in condensed meiotic chromosomes from the grasshopper species *Chorthippus jucundus* after silver staining. **a** Metaphase I spermatocyte. The three larger submetacentric bivalents, as well as the remaining five acro/telocentric bivalents show chromatid cores along their chromosomes. In each bivalent two densely-stained round structures (*arrowheads*), each representing the two closely associated sister kinetochores, appear as core differentiations at homologous centromeres. The univalent telocentric sex chromosome (X) is oriented towards the upper pole. **b** Selected metaphase I sex univalent. The sister cores are separated except at their distal ends. At their proximal end the cores are continuous with the closely associated sister kinetochores that appear as a single structure (*arrowhead*). **c** Selected metaphase I amphitelically oriented telocentric univalent. Sister kinetochores (*arrowheads*) are joined by a silver-stained connecting strand (*arrows*). Sister cores are separated along the arm but joined at their distal end. **d** Selected metaphase I autosomal telocentric bivalent with a single interstitial chiasma. Sister cores are intimately associated between kinetochores and the chiasma site, and between the chiasma site and the distal chromosome ends, while they are individualized at the chiasma. **e** Schematic drawing of an end-view of a cross-section through the arm of one chromosome of bivalent shown in **d**. Each sister chromatid shows a peripherally located core from which chromatin radial loops emanate within a half-circle. **f** Selected submetacentric bivalents with three chiasmata, two in the long arm and one in the short arm, showing the progressive separation of sister cores from early metaphase I up to anaphase I. **g** Anaphase I spermatocyte. The recombined homologues segregate to opposite poles, and the sex chromosome (X) integrates into one pole. All chromosomes present two separated cores continuous with the individualized sister kinetochores (*arrowheads*) at their centromeres. **h** Selected anaphase I amphitelically oriented telocentric univalent. Sister kinetochores (*arrowheads*) are joined by a silver-stained connecting strand (*arrows*). Sister cores are separated at their distal end. **i** Selected anaphase I telocentric chromosome. Each chromatid shows an internal core along its length. Sister kinetochores are discerned. **j** Schematic drawing of an end-view of a cross-section through the arm of the chromosome in **i**. Each chromatid shows an internally located core from which chromatin radial loops emanate within a circle

terstitial chiasma, as illustrated in Fig. 4d, is carefully observed, it becomes evident that there is a single line between the kinetochores and the chiasma, and between the chiasma and distal telomeres (Rufas et al. 1987; Santos et al. 1987). EM observations of serial sections of metaphase I bivalents after cytochemical methods such as the Os-PPD procedure (Antonio et al. 1996), the EDTA regressive staining technique (unpublished results), and silver staining (Suja et al. 1999), reveal that this apparent single line is, in fact, composed of the two parallel and intimately associated cores of both sister chromatids. By light microscopy, the two chromatid cores from each chromosome are only



evident at chiasma sites since they separate at this location (Fig. 4a,d). Due to this characteristic, silver staining has been used to precisely locate and score the number of chiasmata in these highly condensed bivalents (Rufas et al. 1987, 1988), and to support the absence of chiasma terminalization (Santos et al. 1989) and the presence of achiasmatic associations (Santos and Esteban 1990).

If a cross-section through one chromosome region that is not implicated in the chiasma is end-viewed, the sister chromatid cores appear as two associated dots (Fig. 4d,e). Moreover, these dots are located in the middle of the surface of association between sister chromatids. This arrangement implies that the cores are peripherally located with respect to the width of the chromatids (Fig. 4e), i.e. the cores are not completely surrounded by chromatin loops as in metaphase mitotic chromosomes (Fig. 2c). Thus, the metaphase I homologues present a chromosome structure similar to that found during prophase, i.e. they show a meiotic organization (Rufas et al. 1987, 1988; Suja et al. 1991). This organization has also been revealed in metaphase I bivalents of *Lilium*, although in this plant species chromatid cores appear coiled (Stack 1991).

In grasshopper late metaphase I bivalents, chromatid cores individualize (Suja et al. 1991). The chromatid cores begin to separate from their distal ends towards their centromeric ones (Fig. 4f). The separation process proceeds as follows: (i) the cores first disjoin between their distal ends and the chiasma site, although their ends are still associated, (ii) the cores then separate between the chiasma and the centromere with a concomitant individualization of sister kinetochores, and (iii) finally, the connection that persists at their distal ends disappears (Fig. 4f). Therefore, in these late metaphase I bivalents the four chromatid cores are individualized all along their length, and thus occupy an internal position inside each chromatid. Consequently, while in early metaphase I bivalents the chromosomes show a meiotic organization with laterally located cores in each chromatid, in late metaphase I bivalents the chromosomes show a mitotic organization with internally located cores in both sister chromatids (Suja et al. 1991). Interestingly, although late metaphase I chromosomes change their structure, the gross morphology of the bivalents is identical to that observed in early metaphase I. These results indicate that the chromatid cores are not directly involved in maintaining the close association between sister chromatids, i.e. in sister-chromatid cohesion. However, this change of structure from a meiotic to a mitotic organization suggests that it is probably a necessary precondition to progress towards the onset of anaphase I. Anyway, these results point to the sustained presence of cohesion factors between sister chromatids to maintain arm cohesion until the onset of anaphase I (Suja et al. 1992). Recent results obtained after treating grasshopper spermatocytes with colchicine, a well-known microtubule-depolymerizing drug, have shown that in prometaphase I-arrested bivalents the chromatid cores and kinetochores appear individualized, but the homo-

logues remain tightly associated (Rodríguez et al. 2001). This result supports the idea that the change of chromosome structure, from a meiotic to a mitotic one, is independent of tension to opposite poles exerted by bundles of kinetochore microtubules, and that there are cohesive factors that maintain the association between sister chromatids.

The major reorganization of structure that grasshopper chromosomes experience between early and late metaphase I (Suja et al. 1991) has also been reported for mitotic chromosomes (Giménez-Abián et al. 1995). These authors have demonstrated by silver staining that prophase chromosomes appear as cylinders with a single and coiled chromatid core internally located, while during prometaphase this core separates into two coiled ones that will finally occupy an inner location inside each metaphase chromatid. When topo II activity is inhibited and catenations between sister chromatids cannot be resolved, silver staining reveals intimately paired sister cores. Thus, the enzymatic activity of topo II is needed to permit the separation of sister cores during mitotic prometaphase (Giménez-Abián et al. 1995). Therefore, in prophase chromosomes the cores occupy a lateral position on each chromatid and are tightly associated, as occurs in early metaphase I grasshopper chromosomes. Likewise, metaphase mitotic chromatids, as previously explained, show an internally located core, as found in late metaphase I chromosomes.

3.3

Chromatid Cores in Metaphase I Univalents

Most grasshopper species have a sex-determining mechanism of the type XO in males and XX in females. Consequently, the X chromosome in males behaves as a natural univalent, which segregates with both chromatids to a single pole during anaphase I. In metaphase I, and after silver staining, the telocentric X univalents show a prominent dot at one end that represents the closely associated sister kinetochores, as occurs in the autosomes (Fig. 4a,b). Additionally, two separated and parallel sister chromatid cores are observed along the chromosome. These sister cores appear associated at their proximal ends in continuity with sister kinetochores, and at their distal ends (Fig. 4b) (Rufas et al. 1987; Suja et al. 1999; Viera et al. 2004). These distal associations are only resolved by late metaphase I, as occurs in the autosomes (Suja et al. 1991).

The same arrangement and behaviour of sister cores and sister kinetochores has been described in B univalents when they are syntelically oriented, i.e. when both sister kinetochores are connected to the same spindle pole (Suja et al. 1991, 1992, 1999; Viera et al. 2004). B chromosomes are additional dispensable chromosomes that are present in some individuals from certain populations in a wide range of animal and plant species (Jones and Rees 1982).

All these data indicate that separated sister cores are observed when chromosomes have not synapsed during prophase I due to the lack of a homologue. In this respect, when more than two identical B chromosomes are present in the spermatocyte they frequently form bivalents, and in this case their sister cores are closely associated in metaphase I, like those observed in the autosomal bivalents (Suja et al. 1991, 1992).

When B univalents are amphitelically oriented, i.e. when both sister kinetochores are attached to opposite spindle poles, and aligned at the metaphase I plate, their sister cores are separated. However, in these univalents their sister kinetochores are well separated from one another but connected by a silver-stained strand that traverses the centromere region (Fig. 4c) (Suja et al. 1991, 1992), as generally occurs for metaphase II chromosomes (see below). Interestingly, in most instances this connecting strand stretches but does not break during anaphase I.

3.4

Chromatid Cores in Anaphase I and Metaphase II Chromosomes

At the onset of anaphase I, the cohesion present between sister chromatid arms is released, and consequently, the homologues separate and segregate to opposite poles (Suja et al. 1992). In grasshopper anaphase I chromosomes, silver staining demonstrates two individualized sister kinetochores at their centromeres, and an internally located chromatid core along each chromatid (Fig. 4g,i) (Rufas et al. 1987). EM observations of serial sections of these chromosomes after silver staining (our unpublished results) confirm those obtained by light microscopy. Frequently, a round differentiation is found at the distal ends of the chromatid cores in anaphase I chromosomes. Such differentiations, named "telochores", do not contact with the distal chromatid ends since there is always some chromatin beyond them (Suja and Rufas 1994). Following the radial loop/scaffold model of mitotic chromosome structure, it has been proposed that telochores may represent the local accumulation of topo II, which is associated with the bases of the chromatin loops that occupy a hemispherical space at the telomeres (Suja and Rufas 1994).

In grasshopper metaphase II chromosomes, the chromatid cores, as during anaphase I, are internally located inside chromatids appearing either as straight (Suja and Rufas 1994) or coiled longitudinal structures (Nokkala 1985; Nokkala and Nokkala 1986; Zhao et al. 1994). Thus, anaphase I and metaphase II chromosomes present a mitotic organization identical to that found in metaphase mitotic chromosomes (compare Fig. 4j and Fig. 2c).

In addition to the chromatid cores, in grasshopper metaphase II chromosomes, silver staining also reveals that sister kinetochores are separated facing opposite poles and connected by a silver-stained strand (Rufas et al. 1989; Suja et al. 1992). As indicated above, this connecting strand is also discerned in amphitelically oriented B univalents in metaphase I.

However, while a stretched connecting strand persists between sister kinetochores in lagging B univalents during anaphase I, it always disappears during the metaphase II/anaphase II transition. These results support that this connecting strand may represent some protein(s) that associate sister chromatids at the centromere, and would only be released during the metaphase II/anaphase II transition since they are probably protected against degradation during meiosis I (see chapter by Y. Watanabe in this volume).

3.5

Relationship between Chromatid Cores and Lateral Elements

Pachytene and early metaphase I chromosomes show a meiotic organization, but differ in the identity of their axial structures. Is there some relationship between LEs and chromatid cores? These axial structures resemble one another because they are able to be silver-stained, and kinetochores appear as differentiations along them. Moreover, it has been shown that topo II is present at pachytene LEs in rooster spermatocytes (Moens and Earnshaw 1989) and budding yeast meiotic cells (Klein et al. 1992), and at chromatid cores (Earnshaw and Laemmli 1984; Earnshaw et al. 1985). However, although these structures share these characteristics they are not directly related since there is a single LE per pachytene chromosome but two chromatid cores per metaphase I chromosome.

The behaviour of SC elements during post-pachytene stages has been analysed only in some species, but three main strategies have been reported (John 1990): (i) the SC as a tripartite structure disorganizes, but LEs are retained by the disjoined homologues at least in diplotene bivalents of some mammals; (ii) the SC central and lateral elements are extruded as amorphous masses from the diplotene bivalent, and are then degraded in the cytoplasm in *Helix*, *Neottiella* and *Lilium*; and (iii) the SC fragments during diplotene and their elements posteriorly self-assemble in the nucleoplasm or in the cytoplasm as poly-synaptonemal complexes (PSCs) (Goldstein 1987). Grasshopper species follow this last kind of behaviour (Rufas et al. 1992). In this sense, two elegant ultrastructural studies reported that patches of PSCs appeared between the sister chromatids of grasshopper metaphase I bivalents (Esponda and Krimer 1979; Moens and Church 1979). Thus, although it is clear that in grasshoppers the chromatid cores are not related to the SC as a tripartite structure, the behaviour of SCs elements during post-pachytene stages, and the appearance of silver-stained chromatid cores from diakinesis on, led to the proposal of a model whereby the closely associated cores of both sister chromatids could act as a framework for the addition of specific LE proteins (Fig. 3) (Rufas et al. 1992). In this context, the disintegration of the LEs during diplotene, as well as the increasing chromatin condensation, would render the subjacent chromatid cores accessible to silver staining and consequently visible. This model is supported by the observation that on

surface-spread rooster spermatocytes topo II, presumably labelling the chromatid cores, appears preferentially located on the outer edge of the pachytene LEs (Moens and Earnshaw 1989). Other evidence supporting the relationship between chromatid cores and LEs comes from the studies of the SC transformations in diplotene rye microsporocytes (Fedotova et al. 1989). These authors observed by EM that SCs fragmented during diplotene. The gaps between the SC fragments appeared occupied by thin silver-stained threads that resulted from the transformation of the LEs. These threads formed loops and coiled during late diplotene. Thus, it was speculated that these silver-stained threads could be core-like structures. In this context, these coiled core-like structures observed during diplotene were related to the coiled silver-stained chromatid cores observed in lily metaphase I chromosomes (Stack 1991).

A similar model was proposed after the ultrastructural observation of rodent LEs. As a rule, LEs are observed as single lines along each pachytene homologue. However, there are different reports showing that LEs are double in nature since two close and parallel strands are detected along them in both sectioned and spread pachytene spermatocytes (Heyting et al. 1985; Dietrich et al. 1992, and references therein). Given that each homologue is composed of two sister chromatids, it was early proposed that these parallel strands may represent their cores (Wahrman 1981). Dietrich et al. (1992) also reported the presence of an additional third substructure along mouse and rat LEs that was considerably thinner than the other two major strands, and is localized on the inner side of these two major strands of the LE. Taking into account these observations, these authors proposed that the two major strands correspond to the chromatid cores, and that the thinner strand could be responsible for the tight association between the chromatid cores, and would also attach to transverse filaments (Dietrich et al. 1992).

3.6

Correlation between Chromatid Cores, Topo II and Condensin

According to the models presented above, the sister chromatid cores could be closely associated at pachytene LEs, and also until early metaphase I, but which is the mechanism responsible for this association? How is meiotic chromosome structure maintained?

The first indication of the existence of some proteins that could associate sister cores in grasshopper metaphase I bivalents was obtained by using the MPM-2 monoclonal antibody (Suja et al. 1999; Rodríguez et al. 2001). This antibody recognizes different proteins that become phosphorylated at the G2/M transition and that are dephosphorylated at the end of mitosis (Davies et al. 1983). In pachytene spermatocytes MPM-2 delineates SCs, while in metaphase I bivalents the labelling appears at kinetochores, and as a series of small round patches of similar size at the interchromatid domain, which is the surface of contact between sister chromatids (Suja et al. 1999).

At chiasmata regions, where sister cores appear individualized, there is absence of MPM-2 labelling. During the metaphase I/anaphase I transition the MPM-2 phosphoepitopes are released from the interchromatid domain and, in anaphase I chromosomes, MPM-2 only persists at sister kinetochores. Interestingly, in metaphase I univalents, and in prometaphase I-arrested bivalents after colchicine treatment (both situations where sister cores are separated), MPM-2 stains kinetochores but not the interchromatid domain (Suja et al. 1999; Rodríguez et al. 2001). Together, these data indicate that MPM-2 labels some phosphoprotein(s) at kinetochores, and some other protein(s) that might associate sister cores at least during early metaphase I.

There are data supporting the idea that MPM-2 recognizes phosphorylated topo II as a structural component of the chromatid core of isolated mammalian metaphase chromosomes (Taagepera et al. 1993). In this sense, by using an antibody against human topo II on grasshopper spermatocytes, we have obtained a pattern of labelling similar to that found with MPM-2 in both metaphase I autosomal bivalents and univalents (Fig. 5b,d). Interestingly, this antibody does not reveal the chromatid cores along the arms of anaphase I chromosomes. Consequently, there exists the possibility that phosphorylated topo II may associate the sister chromatid cores. However, this possibility leads to the paradox that topo II may be a structural component of the chromatid cores, and may also function as a linker between the sister chromatid cores until early metaphase I. In this sense, it can be argued that there are different chromosomal populations of topo II (Swedlow et al. 1993), which may suffer diverse post-translational modifications to accomplish many aspects of chromosome dynamics. In fact, there is considerable controversy on the role of topo II in chromosome condensation and segregation (for review see Sumner 1996; Warburton and Earnshaw 1997; Paliulis and Nicklas 2003; Swedlow and Hirano 2003; Porter and Farr 2004).

Since topo II is a candidate for maintaining the association between sister cores at the pachytene LE and until early metaphase I, it is reasonable to suppose that the condensin complex, which appears as a component of the chromosome scaffold fraction together with topo II, could also have a similar role. There are few reports describing the distribution of condensin complexes in meiotic chromosomes (for review see Page and Hawley 2004). In this sense, the distribution of condensin complexes in grasshopper meiotic chromosomes is not known. However, results obtained in budding yeast demonstrate that condensin complexes are present at pachytene LEs (Yu and Koshland 2003). Data obtained from budding yeast mutants for the condensin subunits Smc2, Ycg1 and Ycs4 indicate that this complex is necessary for the loading of the structural AE/LE proteins Red1 and Hop1 and SC assembly, for accurate chromosome compaction, pairing, and segregation during the first meiotic division (Yu and Koshland 2003). By contrast, in *Caenorhabditis elegans* meiosis, different condensin subunits appear on chromosomes at diplotene/diakinesis, and mutants for the condensin II subunits HCP-6 and

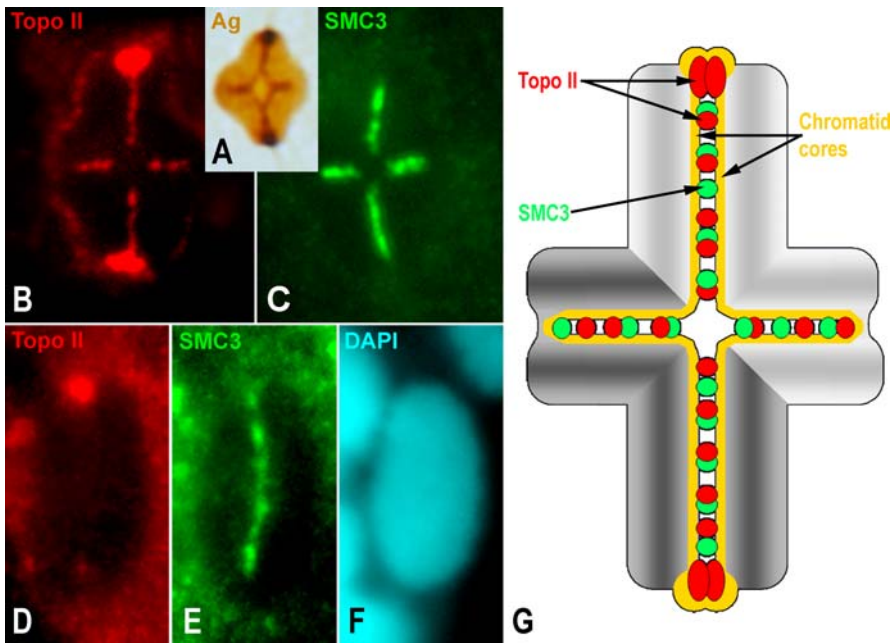


Fig. 5 Selected metaphase I bivalents with a single interstitial chiasma (**a–c**) and univalent X chromosomes (**d–f**) from the grasshopper *Chorthippus jucundus* after silver staining (Ag) (**a**), topo II detection (**b,d**), labelling of the cohesin subunit SMC3 (**c,e**), and DAPI staining of the chromatin (**f**). **a–c** In bivalents, topo II and SMC3 label the interchromatid domain along the arms except at the chiasma region where, by contrast, the silver-stained sister cores appear separated. The centromere regions are only stained by silver and topo II. **d–f** In the univalent X chromosome, topo II is only detected at the centromere, while SMC3 appears at the interchromatid domain. **g** Diagrammatic representation of a telocentric bivalent with a single interstitial chiasma. Sister chromatid cores have been depicted as *separated lines* at the periphery of the chromatids for simplicity. The closely associated sister kinetochores are continuous with the cores. Topo II and the cohesin subunit SMC3 are respectively depicted as *dark and light patches* at the interchromatid domain between the cores. The proximity between the topo II and SMC3 patches does not indicate that they are colocalising. The topo II labelling at centromeres is minimized and superimposed on sister kinetochores

MIX-1 exhibit normal chromosome pairing and formation of SCs, but abnormal chromosome condensation during diakinesis and aberrant chromosome segregation during both meiotic divisions (Chan et al. 2004). These conflicting results clearly indicate that more studies are needed in different species to know the participation of condensin complexes in the formation of axial structures such as the LEs, their possible interaction with topo II and also their participation in the association of sister chromatid cores.

3.7

Relationship between Chromatid Cores and Cohesin Axes

During the last few years, it has been found that an evolutionarily conserved multiprotein complex called cohesin establishes and maintains the association between sister chromatids from DNA replication until anaphase segregation (Miyazaki and Orr-Weaver 1994; Nasmyth 2001; Watanabe 2005). The eukaryotic mitotic cohesin complex consists of two SMC proteins, SMC1 and SMC3, which form an heterodimer, and two non-SMC components called SCC1/RAD21 and SCC3/SA1 or SA2. This complex appears as a ring that may embrace sister chromatids or chromatin loops from sister chromatids, and is differentially released from arms and centromeres in mitosis and meiosis (Losada and Hirano 2005; Nasmyth and Haering 2005). During mitosis, cohesin is also implicated in transcriptional regulation and post-replicative double-strand break repair (for review see Huang and Moazed 2006; Watrin and Peters 2006).

During meiosis, cohesin complexes are detected at centromeres and as patches at the interchromatid domain in metaphase I bivalents. The cohesin complexes at the arms are released at the onset of anaphase I, while those at centromeres are lost during the metaphase II/anaphase II transition (Fig. 1) (see chapter by Y. Watanabe, in this volume, for the molecular mechanisms regulating the release of arm and centromere cohesion). During meiosis, some of the mitotic subunits are replaced or coexist with their meiosis-specific paralogues (Watanabe 2004). In mouse, for instance, SMC1 α coexists with its meiosis-specific paralogue SMC1 β (Revenkova et al. 2001). Likewise, RAD21 is expressed during meiosis (Parra et al. 2004; Xu et al. 2004) as its meiosis-specific paralogue REC8 (Eijpe et al. 2003; Lee et al. 2003). By contrast, SA1 and SA2 seem to be replaced by their meiosis-specific paralogue STAG3 (Prieto et al. 2001). These subunits form several cohesin complexes, which may have different functions in sister-chromatid cohesion according to their different distributions in metaphase I bivalents (Parra et al. 2004; for review see Revenkova and Jessberger 2006). In addition to their role in sister-chromatid cohesion, meiotic cohesin complexes are also required for the monopolar orientation of sister kinetochores during meiosis I, at least in fission yeast, maize and *Arabidopsis thaliana* (Watanabe and Nurse 1999, Hamant et al. 2005; Chelysheva et al. 2005), and probably in mouse (Parra et al. 2004), and are also required to maintain wild-type levels of meiotic recombination. In meiotic yeast mutants for some meiosis-specific cohesin subunits, the conversion of double-strand breaks into crossovers is greatly defective (Klein et al. 1999; Watanabe and Nurse 1999; for review see Nasmyth and Haering 2005).

Cohesin complexes are present as axial structures in prophase I chromosomes. In mouse mutant spermatocytes for SYCP3 and SYCP2, two structural AE/LE proteins, AEs/LEs are not formed, but cohesin complexes with SMC1 α ,

SMC3 and STAG3 persist as axial structures along prophase I chromosomes (Pelttari et al. 2001; Yang et al. 2006). Accordingly, it has been proposed that these “cohesin axes” are required for proper AEs/LEs assembly (Pelttari et al. 2001). These results suggest that the cohesin axes, as well as the associated sister chromatid cores (see above), probably composed by topo II and condensin complexes, could act as frameworks for the addition of AE/LE proteins.

In mouse mutant spermatocytes for SYCP3, the “cohesin axes” are two- to fourfold longer than those of wild-type (Kolas et al. 2004). Thus, AE/LE proteins may have a role in chromosome compaction in mouse (for review see Revenkova and Jessberger 2006). Meiotic cohesin also modulates chromosome compaction during prophase I. In mouse mutant spermatocytes for SMC1 β and REC8, the AEs/LEs form, but they are only half the length of those present in wild-type mice, and their associated chromatin loops are twice as long as in wild-type individuals (Revenkova et al. 2004; Bannister et al. 2004; Xu et al. 2005). By contrast, deletion of the *Rec8* gene results in undercompacted chromosomes, and prevents the formation of AEs and SC in budding yeast (Klein et al. 1999), or linear elements (i.e. chromosome structures related to AEs/LEs) in fission yeast (Molnar et al. 1995). Surprisingly, in fission yeast mutants for the meiosis-specific cohesin subunits Rec8 and Rec11, prophase I chromosomes appear undercompacted, while in mutants for Pds5 (a cohesin-related protein) chromosomes are hypercompacted (Ding et al. 2006). Moreover, *Arabidopsis* Rec8 mutants show abnormal short SC stretches (Chelysheva et al. 2005), and RNAi-mediated Rec8 depletion in *C. elegans* avoids the formation of AEs and SCs (Pasierbek et al. 2001). These apparently conflicting results on the cohesin-dependent formation of AEs/LEs and chromosome compaction may be attributed to the variety and redundancy of cohesin complexes, and their different regulation among organisms. Obviously, the structural and molecular relationships between different cohesin complexes and cohesin-related proteins, AE/LE proteins, and condensin complexes at AEs/LEs during prophase I, and their role in chromosome shaping and condensation, remains to be clarified. In this line, the relationship between cohesin complexes and cohesin-related proteins and topo II in chromosome structure and segregation remains to be elucidated (see Aguilar et al. 2005; Toyoda and Yanagida 2006). However, and taking into account all the above results, it is tempting to propose a working model that implies that the sister chromatid cores from prophase I chromosomes are closely associated by means of a cohesin axis that in turn acts as a framework for the addition of specific AE/LE proteins (Fig. 3). This model is supported by results obtained in *Sordaria macrospora* mutants for Spo76, an orthologue of budding yeast Pds5, a conserved cohesin-related protein. In these mutants, the zygotene AEs frequently split into two thinner half-AEs that do not participate in SC formation (van Heemst et al. 1999). These authors suggested that Spo76 may establish sister-chromatid cohesion in relationship with, but immediately above, the AEs.

The relationship between chromatid cores and cohesin axes in metaphase I chromosomes remains enigmatic. Recently, we have immunolocalized three cohesin subunits in grasshopper metaphase I chromosomes by employing antibodies against human SMC3, *Drosophila* SA1, an orthologue of STAG/SA, and *Drosophila* Rad21 (unpublished results). Our data show that these three cohesin subunits appear at the interchromatid domain in metaphase I autosomal bivalents (Fig. 5c,g), while only SA1 is additionally detected at centromeres. Interestingly, while SMC3 appears at the interchromatid domain in the X (Fig. 5e,f) and B univalents, RAD21 and SA1 are not detected at this domain. Thus, although we cannot exclude the possibility that RAD21 and SA1 are present in small amounts that cannot be detected by antibodies, it seems that these cohesin subunits are not directly responsible for the maintenance of sister-chromatid arm cohesion. In this respect, it is worth mentioning that while SMC3 is detected along AEs from leptotene on, RAD21 and SA1 are only detected at synapsed regions from zygotene. These results indicate that in univalents that have not synapsed, RAD21 and SA1 are not present at their interchromatid domain in metaphase I. Remarkably, as previously described, univalents present separated sister cores. Thus, these observations correlate the lack of synapsis in univalents with the absence of RAD21 and SA1 at their interchromatid domain in metaphase I, and the presence of separated sister cores. Consequently, RAD21 and SA1 could be maintaining the association between sister chromatid cores, but only if synapsis occurs during pachytene. As previously explained, metaphase I univalents show separated sister cores but sister chromatids remain closely associated until anaphase I. Since SMC3 is present at their interchromatid domain in metaphase I, this cohesin subunit would be directly involved in maintaining sister-chromatid arm cohesion. Obviously, these speculations imply that at least two different cohesin complexes may be present during grasshopper meiosis. Moreover, by assuming that sister cores are intimately associated and are part of prophase I LEs, our results indicate that, in grasshopper species, a cohesin complex with SMC3, but not RAD21 and SA1, may associate sister cores from DNA replication and from early prophase I.

4

Concluding Remarks

The data we have presented suggest that the radial loop/scaffold model of mitotic chromosome organization also applies to condensed meiotic chromosomes. In this sense, it is interesting that whereas sister cores individualize during prometaphase in mammalian mitotic chromosomes, in grasshopper meiosis they separate by late metaphase I. These results indicate that chromosome structure changes before segregation during both mitosis and meiosis.

Thus, it is clear that the sister cores do not directly maintain sister-chromatid cohesion. This assumption is supported by the behaviour of the sister cores in metaphase I univalents.

A working model proposes that sister cores are associated by a cohesin axis to which AE/LE proteins attach during prophase I (Fig. 3). However, there is no compelling evidence supporting this model. For instance, the participation of condensin complexes and topo II in the organization of chromatid cores and LEs is still not clear. More studies on the relative distribution and behaviour of topo II, condensin, cohesin and LE proteins in different meiotic systems are needed to understand their possible relationship at these axial structures during prophase I. Likewise, the identity of diverse cohesin complexes at different chromosome domains in metaphase I bivalents remains to be clarified in different meiotic systems. These cohesin complexes may have diverse functions in maintaining the association between sister cores from prophase I up to metaphase I, and in maintaining sister-chromatid arm and centromere cohesion.

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Sister Chromatid Cohesion and Centromere Organization in Meiosis

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Abstract Meiosis is a unique cell cycle that allows halving of the chromosome number, a step essential for sexual reproduction. In meiosis, a single round of DNA replication is followed by two rounds of chromosome segregation that generate four haploid gametes from one diploid cell. To accomplish this specialized chromosome segregation, sister kinetochores are mono-oriented and attached to microtubules emanating from the same spindle pole (monopolar attachment) to segregate homologous chromosomes (rather than sister chromatids) in the first meiotic division (meiosis I). So far, two classes of proteins have been implicated in promoting mono-orientation: meiosis-specific cohesin containing Rec8 and meiosis I-specific kinetochore proteins. Another key regulation during meiosis I involves the removal of sister chromatid cohesion along the arm region, while that around the centromere is retained, since the residual centromeric cohesion is responsible for the faithful segregation of sister kinetochores in the second division (meiosis II). A recently identified family of proteins called shugoshin, which associates with protein phosphatase 2A (PP2A), plays a critical role in the centromeric protection of cohesin at meiosis I.

Abbreviations

APC/c	anaphase-promoting complex/cyclosome
CK1 δ/ϵ	casein kinase 1 δ/ϵ
CPC	chromosomal passenger complex
DSB	double-strand DNA break
HP1	heterochromatin protein 1
PCNA	proliferating cell nuclear antigen
PP2A	protein phosphatase 2A
RFC	replication factor C
SMC	structural maintenance of chromosome

1

Introduction

During the mitotic cell cycle in eukaryotic cells, each chromosome is replicated once in S phase, and the two replication products, called sister chromatids, are evenly segregated to opposite poles during mitosis. Faithful segregation of chromosomes depends on the proper attachment of sister kine-

tochores to the microtubules of the mitotic spindle in the correct orientation (Hauf and Watanabe 2004). To ensure this process, duplicated sister chromatids in S phase are linked together throughout G2 phase until their segregation at anaphase. This linkage is called sister chromatid cohesion and depends on a conserved multisubunit complex, cohesin. During prometaphase, spindle microtubules search for and associate with kinetochores, large proteinaceous structures organized on centromeres acting as an interface for microtubule-chromosome attachment. If both kinetochores on sister chromatids (sister kinetochores) are captured by microtubules from opposite poles (bipolar attachment), then a balance between two forces, one produced by microtubules that pull the sister chromatids apart and the other produced by cohesion that keeps sisters together, emerges. Only when all kinetochores are attached to microtubules and come under tension, are the cohesin complexes holding sister chromatids together removed, thereby allowing the sisters to be segregated to opposite poles by the spindle. This process, called equational segregation, produces two daughter cells that are genetically identical to the mother cell.

Meiosis is a specialized cell cycle that allows halving of the chromosome number, a step for producing haploid gametes (Fig. 1). The meiotic cell cycle consists of a single DNA replication followed by two cycles of nuclear division. During the first meiotic division (meiosis I), homologous chromosomes are segregated, but sisters are partitioned only during the second meiotic division (meiosis II), as in mitosis (Petronczki et al. 2003). Thus, meiosis I has the unique property of chromosome segregation (reductional segregation). To establish meiosis I, at least three processes must be achieved. First, both sister kinetochores within one homolog have to attach to microtubules that emanate from the same spindle pole (monopolar attachment). Second, homologous chromosomes from both parents must pair and recombine, forming chiasmata in which a single sister chromatid from one homolog is covalently attached to one of the sister chromatids from the other homolog. Through the cooperation with sister chromatid cohesion distal to chiasmata, homologous chromosomes are thus physically linked to each other so that the microtubules create tension when they pull homologous chromosomes from opposite directions. Third, sister chromatid cohesion must be removed in a stepwise manner. In order to segregate homologous chromosomes (rather than sisters) during meiosis I, only arm cohesion distal to the chiasmata must be removed, while centromeric cohesion must be retained until meiosis II, when equational segregation occurs with the dissociation of the residual centromeric cohesion.

Although these processes are highly conserved among eukaryotes, our knowledge about the mechanisms is limited. Here, we summarize recent progress of studies that shed light on the molecular bases of establishing monopolar attachment and protecting centromeric cohesion during meiosis I.

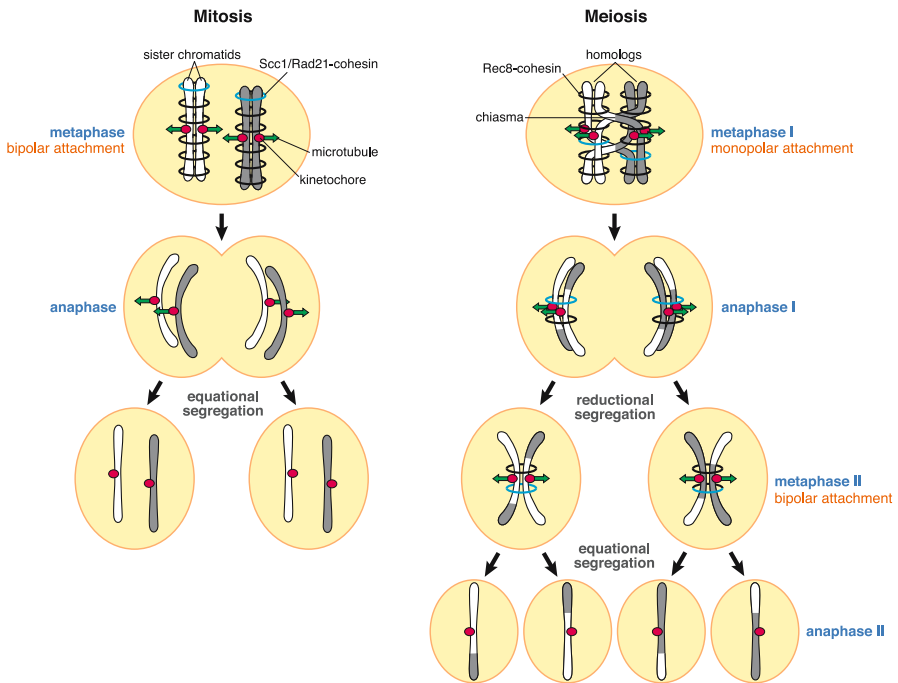


Fig. 1 Chromosome segregation in mitosis and meiosis. In mitotic cell cycle, sister kinetochores are captured by microtubules from opposite poles. Sister chromatid cohesion mediated by Scc1/Rad21-cohesin is removed from the entire chromosome length at anaphase, which allows sister chromatids to be segregated to opposite poles and thus makes two daughter cells genetically identical to the mother cell. During meiosis, Rec8 substitutes for the Scc1/Rad21-kleisin subunit to form a meiosis-specific cohesin complex. Homologous chromosomes from both parents are covalently attached to one of sister chromatids from the other homolog by chiasmata as a result of recombination. During meiosis I, both sister kinetochores attach to microtubules that emanate from the same spindle pole, whereas homologous chromosomes are attached from the opposite poles and thereby become segregated. During meiosis II, sister kinetochores are partitioned as in mitosis. Remarkably, sister chromatids cohesion is removed in a stepwise manner, first along chromosome arms at meiosis I and second in the vicinity of centromeres at meiosis II

2

Cohesin Complex and Sister Chromatid Cohesion

2.1

In Mitosis

The cohesin complex mediates cohesion between sister chromatids during both mitosis and meiosis. The mitotic cohesin complex consists of four core subunits: two SMC (structural maintenance of chromosome) family proteins, Smc1

and Smc3; a kleisin family protein, Scc1 (also called Mcd1 in budding yeast *Saccharomyces cerevisiae* and Rad21 in fission yeast *Schizosaccharomyces pombe*); and Scc3 (Nasmyth and Haering 2005) (Fig. 2). A fifth protein, Pds5, appears to bind less tightly to the cohesin complex. Recent studies suggest that the Smc1 and Smc3 proteins dimerize via their central domains, the so-called hinge domains, to form V-shaped molecules whose arms are composed of coiled-coils and whose apices are composed of the ATPase-containing head domains. The kleisin subunit Scc1 connects the two ATPase head domains of the Smc1–Smc3 heterodimer, thereby forming a large proteinaceous ring. Scc3 interacts with the cohesin complex through its binding to Scc1. It is proposed that the cohesin complex mediates sister chromatid cohesion by directly embracing sister chromatids within the ring (Haering and Nasmyth 2003).

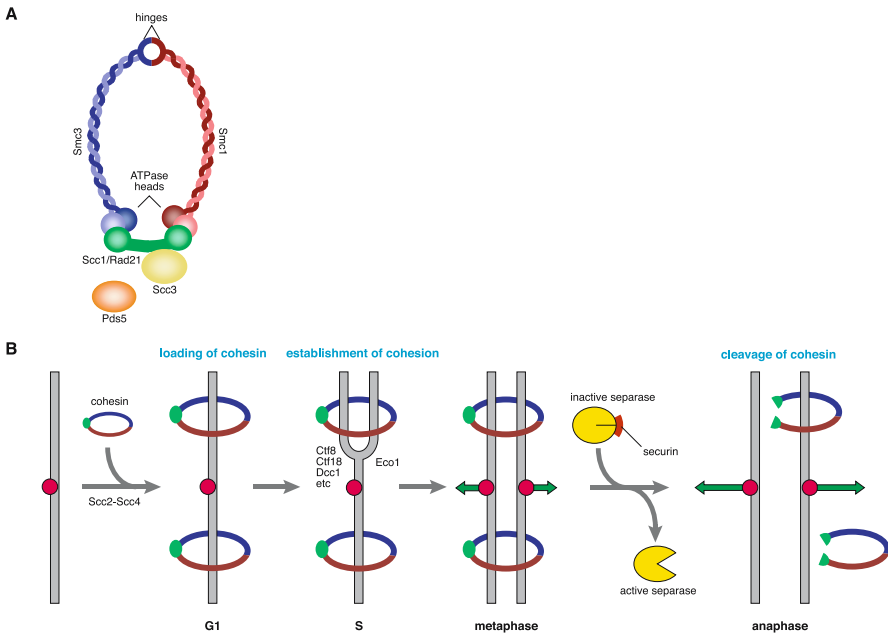


Fig. 2 Sister chromatid cohesion is mediated by cohesin. **A** Schematic model of cohesin complex. ATPase head domains of Smc1–Smc3 dimer are bound by different ends of the Scc1/Rad21 kleisin subunit, thereby forming a large proteinaceous ring. **B** The regulation of sister chromatid cohesion. Before S phase of cell cycle, cohesin complex loads onto chromosomes depending on the function of cohesin loading factor, Scc2–Scc4. Sister chromatid cohesion is established during S phase with the aid of several proteins, including the Eco1 acetyl transferase and RFC containing Ctf8, Ctf18, and Dcc1. When both kinetochores on sister chromatids are captured by microtubules from opposite poles at metaphase, the tension is generated between the pulling force of microtubules and sister chromatid cohesion. After all kinetochores are attached to microtubules and come under tension, destruction of securin by the APC/c activates separase. Cleavage of the Scc1/Rad21-kleisin subunit by separase triggers the dissociation of cohesin from chromosomes and resolves cohesion at the metaphase to anaphase transition

The cohesin complex loads onto chromosomes depending on the function of cohesin loading factor, Scc2–Scc4 (Ciosk et al. 2000) (Fig. 2B). Cohesins can load onto chromosomes throughout the cell cycle in an Scc2–Scc4-dependent manner, but those loaded before S phase are essential and sufficient for producing sister chromatid cohesion (Lengronne et al. 2006) since cohesion is established only during S phase with the aid of Eco1 acetyl transferase (also called Ctf7) (Ivanov et al. 2002; Skibbens et al. 1999; Toth et al. 1999). Another group of proteins, including an alternative replication factor C (RFC) complex that contains Ctf8, Ctf18 and Dcc1 as specific subunits, a putative DNA helicase called Chl1, a DNA polymerase alpha-associated protein called Ctf4, a chromosome remodeling factor complex called RSC, and a replication-pausing checkpoint complex Tof1–Mrc1–Csm3, also appear to be important (but not essential) for the efficient production of sister chromatid cohesion in budding yeast (Hanna et al. 2001; Kenna and Skibbens 2003; Mayer et al. 2001; 2004; Petronczki et al. 2004; Skibbens 2004; Xu et al. 2004). These factors show striking similarity to parts of the DNA replication machinery, suggesting that cohesion is established between sister chromatids as they emerge from the replication forks. Indeed, Eco1 binds directly to the proliferating cell nuclear antigen (PCNA), a ring-shaped cofactor of DNA polymerases, and this association is essential for the establishment of cohesion (Moldovan et al. 2006), suggesting that the role of RFC (Ctf8, Ctf18, and Dcc1) probably is the loading of PCNA onto chromosomes. This may be important for the efficient recruitment of Eco1 at the replication forks. However, little is known about the molecular bases for the establishment of sister chromatid cohesion, including whether Eco1 has an acetyl transferase activity *in vivo* and, if so, the nature of its physiological substrate. Remarkably, like the cohesin complex, most of the factors involved in the loading and the establishment of sister chromatid cohesion are evolutionarily conserved among eukaryotes (Table 1).

Cohesion between sister chromatids is maintained throughout G2 phase until metaphase (Fig. 2B). Sister chromatid cohesion ensures that the tension necessary to stabilize kinetochore-associated microtubules is generated when sister kinetochores have attached only in a bipolar manner (Tanaka 2005). A regulatory mechanism called the spindle assembly checkpoint, a system surveying the proper kinetochore-microtubule attachment and tension, blocks sister chromatid separation until all sister kinetochores are attached to microtubules emanating from the opposite spindle poles, and the forces of the microtubules pulling the sister kinetochores outward and the cohesion keeping the sister chromatids together comes into balance. Chromatid disjunction at anaphase is triggered by the activation of a thiol-protease called separase (Esp1 in *S. cerevisiae* and Cut1 in *S. pombe*), which cleaves kleisin subunit Scc1 of cohesin, thereby opening the cohesin ring to release sister chromatid cohesion (Nasmyth 2001; Uhlmann 2003). Separase is kept inhibited through its association with an inhibitory chaperone called securin (Pds1 in *S. cerevisiae* and Cut2 in *S. pombe*). When the spindle assembly checkpoint has been

Table 1 Cohesin and sister chromatid cohesion-regulating factors in various organisms

	<i>S. cerevisiae</i> Mitosis	Meiosis ^a	<i>S. pombe</i> Mitosis	Meiosis	<i>D. melanogaster</i> Mitosis	Meiosis	<i>H. sapiens</i> Mitosis	Meiosis
Cohesin	Smc1 Smc3 Scc1 (Mcd1) Scc3 (Irr1) Pds5 Scc2 Scc4 Eco1 (Ctf7)	Rec8	Psm1 Psm3 Rad21 Psc3 Pds5 Mis4 Ssl3 Eso1 Cut1 Cut2 Sgo2	Rec8 Rec11	dSmc1 dSmc3 (dCap) dRad21 SA dPds5 Nipped-B CG4203 dEco Sse Pim Mei-S332	C(2)M ^b SA-2	Smc1 Smc3 Scc1 (hHR23A) SA1, SA2 Pds5A, Pds5B Scc2 (NIPBL) Scc4 (hMAU-2) Efo2, Esco2 Separase PTTG1 hSgo1, hSgo2	Smc1β Rec8 SA3
Separase	Esp1							
Securin	Pds1							
Shugoshin	Sgo1			Sgo1				

^a Meiosis-specific counterpart

^b Role for sister chromatid cohesion is unclear

turned off, securin is targeted for proteolytic destruction by an ubiquitin protein ligase called the anaphase-promoting complex/cyclosome (APC/c) and its activator Cdc20.

Although these principles of sister chromatid cohesion and its regulation are presumably applicable to all chromosomes of all eukaryotic organisms, there are some modifications depending on the regions of the chromosome and depending on whether it occurs during mitosis or meiosis. For example, in vertebrate cells, the majority of cohesin complexes dissociates from chromosome arms when the cell enters mitotic prophase, resulting in the formation of an X-shaped mitotic chromosome. This activity called “prophase pathway” is independent from Scc1/Rad21 cleavage but requires two mitotic kinases, Plk1 and Aurora B, and a cohesin-interacting protein Wapl (Gandhi et al. 2006; Kueng et al. 2006; Losada et al. 2002; Sumara et al. 2002). Centromeric cohesin is resistant to prophase pathway activity and is removed mainly by cleavage of the Scc1/Rad21-kleisin subunit by separase at the metaphase-to-anaphase transition (Hauf et al. 2001).

In addition to the cohesion for chromosome segregation, cohesin has an important role for double-strand break (DSB) repair, probably by tethering both broken ends in physical proximity to perform efficient homologous recombination reactions. Recently it was reported that DSB induction not only elicits chromosomal recruitment of cohesin but also establishes chromatid cohesion at the position of DSB (Ström and Sjögren 2005). Although this process might be also essential for meiotic recombination, it is still unclear how the establishment of cohesion in response to DNA damage is managed. Similarly, sister chromatid cohesion mediated by cohesin around rDNA repeats plays an important role in preventing unequal sister chromatid recombination, contributing to the regulation of copy number of rDNA repeats during cell division (Kobayashi et al. 2004).

2.2

In Meiosis

In the meiotic cohesin complex, a meiosis-specific kleisin subunit, Rec8, substitutes for most of Scc1/Rad21, although Scc1/Rad21 is not completely removed from meiotic chromosomes. In the absence of Rec8, meiosis-specific chromosome events such as synaptonemal complex formation, reciprocal recombination between homologous non-sister chromatids, monopolar attachment of sister chromatids (at least in plants and fission yeast), and the protection of centromeric cohesion are all abolished (Chelysheva et al. 2005; DeVeaux and Smith, 1994; Klein et al. 1999; Parisi et al. 1999; Watanabe and Nurse, 1999; Yu and Dawe, 2000). Remarkably, these meiosis-specific properties of chromosomes are not restored by the ectopic expression of Scc1/Rad21 in *rec8* mutant cells even though sister chromatid cohesion is established by Scc1/Rad21-cohesin (Toth et al. 2000; Yokobayashi et al. 2003).

Thus, the meiosis-specific cohesin appears to influence other aspects of chromosome behavior in addition to cohesion, perhaps including the loading of Spo11/Rec12 (see Cromie and Smith, this series). If meiosis is induced ectopically after mitotic DNA replication by activating the meiosis-initiation network, Rec8-cohesin is expressed and associates with chromatin but fails to execute meiotic function (Watanabe et al. 2001). It is most likely that this meiotic cohesin does not participate in sister chromatid cohesion because cohesin can produce cohesion only during S phase (Uhlmann and Nasmyth 1998). Therefore, the qualitative change from a mitotic chromosome to a meiotic chromosome arises before the premeiotic S phase when the exchange of mitotic cohesin for meiotic cohesin occurs.

Additional meiosis-specific cohesin subunits have been found in various organisms (Table 1). Fission yeast contains two Scc3 homologs, Psc3 and Rec11. Psc3 is expressed both in mitosis and meiosis, and interacts with Rad21-cohesin and Rec8-cohesin. On the other hand, Rec11 is expressed specifically in meiosis and forms a complex with Rec8-cohesin. Interestingly, Rec8-cohesin along chromosome arms contains Rec11, whereas that in the vicinity of centromeres contains Psc3 (Kitajima et al. 2003b). The selective inactivation of Rec11 or Psc3 at meiosis can distinguish cohesin functions at centromeres and along the arm regions. The inactivation of Psc3 during meiosis does not influence arm cohesion, but impairs both monopolar attachment and the protection of centromeric cohesion at meiosis I. Actually, the Rec8–Psc3 cohesin consists of two different types of assemblies in the centromeric region. First, the Rec8–Psc3 cohesin localizing at the central core region is specifically required for establishing monopolar attachment at meiosis I. Second, the preferential localization of Rec8–Psc3 cohesin at the pericentromeric repeats is required for cohesion during meiosis II, and is protected from destruction at meiosis I (Watanabe, 2005). The recruitment of Rec8–Psc3 cohesin to the pericentromeric region depends on the heterochromatin structure and Swi6, a fission yeast homolog of mammalian heterochromatin protein 1 (HP1), which has the ability to interact with Psc3. In contrast, in *rec11* mutant cells, centromeric cohesion is preserved, but arm cohesion is separated precociously, with impaired recombination. Consequently, *rec11* cells induce the non-disjunction of homologous chromosomes at meiosis I, illuminating the importance of chiasmata formation by recombination and sister chromatid cohesion along the chromosome arm region for the ensuring homolog segregation.

3

Monopolar Attachment at Meiosis I

During mitosis or meiosis II, the back-to-back configuration of sister kinetochores may facilitate bipolar attachment, since the attachment of one kineto-

chore to one pole orients the other kinetochore toward the opposite pole. In contrast, sister kinetochores orient side-by-side in meiosis I to orient them to the same pole. Although the mechanisms for kinetochore-microtubule attachment are presumably the same in any type of chromosome segregation, there is some additional regulation for suppressing sister kinetochore bi-orientation at meiosis I. So far, two classes of proteins have been implicated in promoting mono-orientation: meiosis-specific cohesin containing Rec8-kleisin subunit and meiosis I-specific kinetochore proteins. Although the molecular bases for organizing mono-orientation have been mainly studied in fission yeast and budding yeast, a generalized view of the regulation of monopolar attachment has not yet been drawn.

3.1

Regulation of Monopolar Attachment in Fission Yeast

An important clue linking cohesin complexes to kinetochore orientation came from the finding that the meiosis-specific kleisin subunit of cohesin, Rec8, is essential for the mono-orientation of sister kinetochores at meiosis I (Watanabe and Nurse, 1999). Similarly, it was recently reported that the Rec8 and Scc3 proteins are essential for the monopolar attachment of sister kinetochores in *Arabidopsis thaliana* meiosis (Chelysheva et al. 2005). When Rec8 is replaced by its mitotic counterpart Rad21, sister chromatids stay closely connected until anaphase of meiosis I. However, these cells fail to establish monopolar attachment of sister kinetochores, and undergo equational rather than reductional division at meiosis I. This equational segregation at meiosis I depends on the function of Rad21, which relocates to the centromeres and establishes centromeric cohesion if Rec8 is absent (Yokobayashi et al. 2003). This suggests that only Rec8-cohesin, but not Rad21-cohesin, enables the establishment of monopolar attachment at meiosis I. Analysis of the localization of cohesins on chromosomes demonstrated that Rad21-cohesins localize to the chromosome arms and pericentromeric heterochromatin, but not to the central core region of the centromere, similar to their localization in mitosis. In contrast, Rec8-cohesins additionally localize to the central core region of the centromere. In mitosis, the enrichment of Rad21-cohesins at the pericentromeric region depends on the formation of heterochromatin (Bernard et al. 2001b; Nonaka et al. 2002). Likewise, heterochromatin is also required in meiosis for the recruitment of Rec8-cohesins to pericentromeric regions, but not to the central core regions. Importantly, a lack of heterochromatin specifically abolishes the centromeric cohesion that persists during anaphase of meiosis I, but does not affect monopolar attachment (Kitajima et al. 2003b). Similarly, shugoshin Sgo1, which localizes exclusively in the heterochromatic region and has a role in protecting centromeric cohesion during anaphase I, is dispensable for monopolar attachment (Kitajima et al. 2004; Rabitsch et al. 2004; see below). These data indicate that the cohesion be-

tween sister chromatids at meiosis I depends on Rec8-cohesins localizing to the pericentromeric region, whereas the mono-orientation of sister kinetochores is carried out by Rec8-cohesins recruited to the central core region. In fission yeast, the centromeric core region is located between inverted repeats that are nearly identical to each other. It has, therefore, been proposed that the central core part of the centromere loops out from the peripheral pericentromeric region (Takahashi et al. 1992). Moreover, the central core region is the site of the assembly of the kinetochore, where spindle microtubules attach (Nakaseko et al. 2001). These findings suggest one attractive hypothesis that the Rec8-cohesins establish cohesion between centromeric core regions of sister chromatids to organize the side-by-side orientation of sister kinetochores

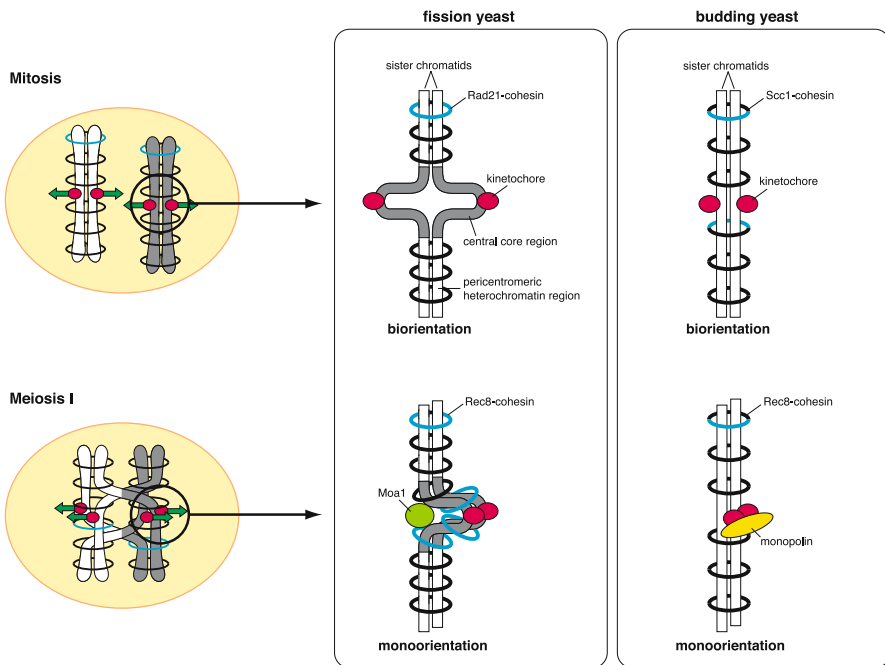


Fig. 3 Regulation of monopolar attachment at meiosis I in fission yeast and budding yeast. In fission yeast, it has been proposed that the central core part of the centromere loops out from the peripheral pericentromeric region. Rad21-cohesin localizes to the pericentromeric heterochromatin, but not to the central core region of the centromere. In contrast, Rec8-cohesin localizes to both pericentromeric heterochromatin and the central core region. Cohesion between sister chromatids at meiosis I relies on Rec8-cohesins localizing at pericentromeric region, whereas monoorientation of sister kinetochores is carried out by Rec8-cohesins recruited to the central core region. Presumably, Moa1 has a role in facilitating central core cohesion by Rec8-cohesins. Rec8-mediated cohesion is not specifically involved in establishing monoorientation of kinetochores in budding yeast. Instead, the monopolin complex including Mam1, Csm1, Lrs4 and Hrr25, is essential for monopolar attachment at meiosis I

(Watanabe et al. 2001; Yokobayashi et al. 2003) (Fig. 3). To assess this hypothesis, Rec8-dependent cohesion was regionally disrupted at the centromeric central core region along chromosomes, resulting in equational segregation at meiosis I (while leaving sister cohesion intact in other chromosomal regions). This result clearly proves that the mono-orientation of sister kinetochores is regulated by Rec8-mediated cohesion at the specific region of centromere (Yokobayashi and Watanabe, 2005). Thus, centromeric Rec8-cohesins seem to execute two region-specific functions, both of which depend on the establishment of cohesion: central core cohesion promotes mono-orientation of sister kinetochores, whereas pericentromeric cohesion holds sister chromatids together until meiosis II.

The Rec8 localizing central core region is essential for establishing mono-orientation, but it is not sufficient. Ectopic expression of Rec8 in mitotic cells does not induce mono-orientation, although Rec8-cohesins do localize at the central core region of centromeres. Other meiosis-specific factors that aid Rec8 in establishing mono-orientation must, therefore, exist. Genetic screening in fission yeast has revealed one such factor named Moa1 (Yokobayashi and Watanabe, 2005). Moa1 is a meiosis-specific kinetochore protein that localizes exclusively at the central core of the centromere from premeiotic S phase to metaphase I, but disappears in anaphase I. In the absence of Moa1, sister chromatids frequently move to opposite poles in anaphase I, as in cells where central core Rec8 is disrupted. Moa1 physically interacts with Rec8, implying that Moa1 functions through Rec8. Presumably, Moa1 has a role in facilitating central core cohesion by Rec8-cohesins, but the detailed mechanism remains elusive. In addition, since the simultaneous expression of Moa1 and Rec8 in mitosis is still not sufficient for mono-orientation, other factors or regulators may remain to be discovered.

3.2

Regulation of Monopolar Attachment in Budding Yeast

Unlike the centromeres of fission yeast and other eukaryotes, the budding yeast centromere is unique in comprising a short stretch of DNA that is attached to a single microtubule and lacks pericentromeric heterochromatin. The involvement of cohesion in the regulation of mono-orientation is not clear in this organism. In contrast to fission yeast and plants, both Rec8- and Scc1/Rad21-cohesins are recruited to the central core of centromeres, and both cohesins can support mono-orientation in meiosis I (Riedel et al. 2006; Toth et al. 2000). Therefore, Rec8-mediated cohesion is not specifically involved in establishing the mono-orientation of kinetochores in budding yeast. Instead, a different set of meiosis-specific kinetochore protein complexes called monopolin, which includes Mam1, Csm1, Lrs4 and Hrr25, is essential for monopolar attachment at meiosis I (Petronczki et al. 2006; Rabitsch et al. 2003; Toth et al. 2000) (Fig. 3). Although Mam1 is a meiosis-specific

protein, Csm1 and Lrs4 form a complex with nucleolar proteins in mitosis, which is released from the nucleolus at metaphase I and recruited to kinetochores together with Mam1. Hrr25, a conserved casein kinase 1 δ/ϵ (CK1 δ/ϵ) homolog in budding yeast, has multiple functions in many different cellular processes, but is recruited to monopolin by associating with Mam1 at meiosis I, and promotes mono-orientation. Monopolin localizes to kinetochores from prophase I to metaphase I and is not present thereafter. This localization to the kinetochore requires the Polo-like kinase Cdc5 (Clyne et al. 2003; Lee and Amon 2003). Cdc5 promotes the release of Lrs4 from nucleoli during prophase I, and may also regulate monopolin function through the phosphorylation of Mam1. Cdc5 is also required for the formation of chiasmata and the phosphorylation of Rec8 to remove cohesion in a stepwise manner during meiosis (see below). Recruitment of the monopolin complex to kinetochores also requires Spo13, a meiosis-specific protein localizing in the centromere-surrounding regions (Katis et al. 2004b; Lee et al. 2004). Spo13 has a double role in meiosis I, since it is not only required for mono-orientation, but also for the maintenance of centromeric cohesion during meiosis I.

The underlying mechanism, through which monopolin suppresses bi-orientation, has not yet been revealed. It is possible that some kinetochore proteins are phosphorylated by Hrr25 CK1 δ/ϵ which modifies their kinetochore function to prevent bi-orientation during meiosis I. The findings that Hrr25 associates with and phosphorylates Rec8 are particularly interesting, as Rec8 has been implicated in the mono-orientation process in fission yeast and plants (Chelysheva et al. 2005; Watanabe and Nurse 1999; Yu and Dawe 2000). One simple and attractive model is that the phosphorylation of Rec8 at centromeres by Hrr25 converts the ability of Rec8 in a manner that facilitates mono-orientation. However, the story is not so simple. Hrr25 associates with Rec8-, but not with Scc1-cohesin, although both types of cohesin can support mono-orientation mediated by Hrr25. In addition, Hrr25-dependent phosphorylation is not specific for centromeric Rec8, and the phosphorylation status of the majority of the cellular pool of Rec8 is affected by Hrr25 inactivation. Yet it is still possible that the phosphorylation of centromeric cohesin (whether it contains Scc1 or Rec8) by Hrr25 is important for mono-orientation.

It is very difficult to find any similarity between fission yeast Moa1 and subunits of the monopolin complex. Moreover, orthologs of the monopolin subunits (except Hrr25) and Spo13 have so far not been identified in other organisms. One of the monopolin subunits, Csm1, shows limited similarity to the fission yeast Pcs1. However, Pcs1 is required for chromosome segregation during mitosis and meiosis II, but not for meiosis I, indicating that the function of Csm1 in the mono-orientation of sister kinetochores is not conserved in fission yeast (Rabitsch et al. 2003). Fission yeast has two CK1 δ/ϵ (Hrr25) homologs called Hhp1 and Hhp2. In fission yeast, CK1 δ/ϵ activity is also important for accurate chromosome segregation during meiosis I but the physi-

ological significance of their requirement for mono-orientation remains to be analyzed in detail (Petronczki et al. 2006). Thus, it is conceivable that budding yeast employs different mechanisms from fission yeast or metazoans to establish monopolar sister kinetochores, due to the smaller size of its centromere. However, it is still tenable that the monopolin complex regulates cohesin at centromeres to promote the fusion of the sister kinetochores, which might be the ultimate output to promote mono-orientation, since a foldback structure model for budding yeast kinetochore that is very similar to that for fission yeast kinetochore has been proposed recently (Bloom et al. 2006).

4

Stepwise Loss of Cohesion

In meiosis, two rounds of chromosome segregation occur after a single round of DNA replication. Because the homologous chromosomes are paired and joined as a result of recombination, cohesion not only binds the sister chromatids, but also links all four homologous chromatids together at prophase I (Fig. 1). Disjunction of homologous chromosomes during meiosis I depends on the removal of chromosome arm cohesion distal to the chiasmata. To achieve this, cohesins along the entire arm regions are removed at the onset of anaphase I. However, unlike in mitosis, where all cohesins are removed along the entire chromosome at the metaphase-to-anaphase transition, cohesins around the centromere are preserved throughout meiosis I. This residual centromeric cohesion ensures the attachment of sister kinetochores with the meiosis II spindles emanating from opposite poles, thereby sister kinetochores segregate equationally at anaphase II. Thus, in order to perform successive chromosome segregations during meiosis, sister chromatid cohesion, which is established only once during premeiotic DNA replication, is utilized twice by being regionally dissociated in a stepwise manner.

In both budding yeast and fission yeast, the expression of a non-cleavable Rec8 that lacks the separase cleavage sites blocks homologous chromosome segregation at meiosis I. Indeed, suppression of the recombination process, which abolishes the formation of chiasmata, restores this segregation block (Buonomo et al. 2000; Kitajima et al. 2003a). Moreover, if the cohesin along the chromosome arm is abolished by depleting Rec11, the arm-specific meiotic Scc3 subunit in fission yeast, the inability to undergo homolog separation induced by non-cleavable Rec8, is again eliminated. In this situation, however, sister kinetochores, which are still linked via cohesion around the centromere, remain tightly associated, and their separation is blocked at meiosis II. This blockage is released by the inactivation of Psc3, a centromeric counterpart of Rec11/Scc3 (Kitajima et al. 2003a). These observations lead to two important conclusions: first, that meiosis-specific kleisin Rec8 is also cleaved by separase, the same protease that cleaves mitotic kleisin

Scc1/Rad21; second, the cleavage of Rec8 along the chromosome arms leads the chiasmata to segregate homologous chromosomes at meiosis I, whereas Rec8 cleavage around the centromeres is required for the disjunction of sister kinetochores at meiosis II.

4.1

Protection of Centromeric Cohesion at Meiosis I

If Scc1/Rad21 is expressed during meiosis instead of Rec8, all cohesion between sister chromatids is removed at the onset of metaphase-to-anaphase transition in meiosis I. Thus, the ability of centromeric Rec8 to resist cleavage by separase at meiosis I is not possessed by its mitotic counterpart, Scc1/Rad21. Why is centromeric Rec8 cleaved only at meiosis II but not at meiosis I? One prediction was the existence of a meiosis-specific kinetochore factor that protects Rec8 from separase only during meiosis I.

A protein with this feature was identified in functional or knockout screening in yeast and named shugoshin, which means “guardian spirit” in Japanese (Kitajima et al. 2004; Marston et al. 2004; Rabitsch et al. 2004) (Fig. 4). It turns out that shugoshin shares a hitherto unperceived similarity to Mei-S332, a kinetochore protein essential for preventing the loss of centromeric cohesion during meiosis I in *Drosophila melanogaster* (Kerrebrock et al. 1995; Lee and Orr-Weaver 2001), and constitutes a conserved protein family in eukaryotes. Fission yeast possesses two paralogs of the shugoshin family, one called Sgo1, which is expressed exclusively during meiosis I and is essential for the protection of centromeric cohesion, and the other called Sgo2, which is expressed in both mitotic and meiotic cells and is not required for preserving centromeric Rec8 (although it has an important role in accurate chromosome segregation during meiosis). The ectopic expression of Sgo1 together with Rec8 in mitotic cells inhibits the degradation of centromeric Rec8 at anaphase, resulting in the blockage of sister chromatid separation. This protection is relatively specific to Rec8 because the enforced expression of Sgo1 has less effect on Rad21-dependent cohesion (Kitajima et al. 2004). Usually, Sgo1 localizes exclusively at the heterochromatic region from prophase to metaphase in meiosis I, but disappears quickly after anaphase I. As the heterochromatin-dependent enrichment of Rec8-cohesin around centromeres is important for persisting centromeric cohesion, these observations suggest that Sgo1 protects pericentromeric Rec8 from cleavage by separase at the onset of anaphase I. Indeed, Sgo1-depleted cells lose their centromeric Rec8-cohesin during anaphase I, resulting in the precocious separation of sister kinetochores prior to metaphase II (Kitajima et al. 2004; Rabitsch et al. 2004). This causes random chromatid segregation at the ensuing meiosis II. Although Sgo1-depleted cells lose centromeric cohesion during anaphase I, monopolar kinetochore orientation is preserved, which is why the segregation defect does not occur until meiosis II. Budding yeast, flies, and worms

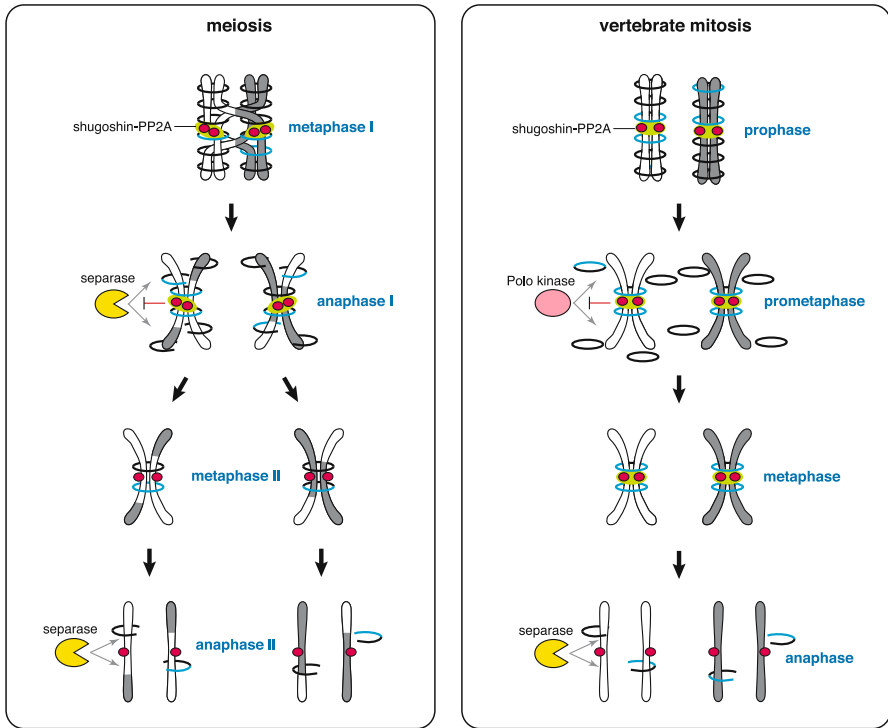


Fig. 4 Shugoshin-PP2A complex protects centromeric cohesin. At the onset of anaphase I, shugoshin-PP2A complex protects centromeric Rec8-cohesin from separase cleavage. This allows the segregation of homologous chromosomes preserving centromeric cohesion between sister kinetochores until meiosis II. At the onset of anaphase II, the residual Rec8 kleisin subunits are cleaved, resulting in the equational segregation of sister kinetochores. In vertebrates, shugoshin-PP2A also protects centromeric cohesin in mitosis. During prophase of proliferating cells, Polo kinase acts to dissociate cohesins from chromosome arms by a mechanism called prophase pathway via the phosphorylation of cohesin's SA2 subunit. Shugoshin-PP2A protects centromeric cohesin from prophase dissociation by inverting the phosphorylation state of SA2 subunit

possess only a single ortholog of the shugoshin protein family that is expressed in mitotic as well as meiotic cells. Budding yeast Sgo1 plays the parts of both fission yeast Sgo1 and Sgo2, maintains centromeric cohesin during meiosis I, and maintains kinetochore functions required for proper chromosome segregation (Katis et al. 2004a; Kitajima et al. 2004; Marston et al. 2004).

The presence of Sgo1 family proteins in mitosis or during meiosis II in budding yeast and *Drosophila* suggests the possibility that these proteins do not participate alone in the protection of centromeric Rec8-kleisin. This speculation is consistent with the observation that the enforced expression of fission yeast Sgo1 during meiosis II does not block the disjunction of

sister kinetochores. Thus, yet other meiosis I-specific factors that confer centromeric protection together with Sgo1 during meiosis I might exist. The protein phosphatase 2A (PP2A) complex was identified as such a factor (Kitajima et al. 2006; Riedel et al. 2006). PP2A is known to act as a heterotrimeric complex containing catalytic (C), scaffold (A) and variable regulatory (B, B', B'' and B''') subunits. Sgo1 proteins in both fission and budding yeast associate only with a specialized type of PP2A complex consisting of a C–A–B' combination and recruit it to centromeres at meiosis I. The inactivation of PP2A also causes meiotic phenotypes, the failure to protect centromeric cohesin at anaphase I, and random segregation of sister chromatids at meiosis II. These effects resemble those of Sgo1 deletion. Importantly, the artificial recruitment of PP2A to the chromosome arms dephosphorylates Rec8 and blocks the resolution of chiasmata at meiosis I in fission yeast. Moreover, the artificial localization of the PP2A complex in pericentromeric heterochromatin regions, the sites where Sgo1 usually localizes at meiosis I, strongly inhibits sister chromatid disjunction in mitotic cells in a manner depending on Rec8 but not Sgo1. These results demonstrate that the efficient cleavage of Rec8 by separase requires the phosphorylation of cohesin, and that this is antagonized by PP2A recruited to centromeres by Sgo1 at meiosis I.

In budding yeast, the Scc1/Rad21 subunit of cohesin is phosphorylated by a Polo-like kinase Cdc5 in mitosis, which is important for efficient Scc1/Rad21 cleavage by separase (Alexandru et al. 2001; Hornig and Uhlmann, 2004). Likewise, Cdc5 is required for the phosphorylation of the Rec8 kleisin subunit and the removal of meiotic cohesin from chromosomes (Clyne et al. 2003; Lee and Amon, 2003). Indeed, cells expressing the *rec8-17A* mutant, in which 14 Cdc5-dependent and three Cdc5-independent phosphorylation sites in Rec8 are replaced by alanines, appears to be delayed in metaphase I due to a Rec8 cleavage defect (Brar et al. 2006). These observations are consistent with the notion that the centromeric Sgo1–PP2A dephosphorylates Rec8, which is phosphorylated by the Polo-like kinase, to protect centromeric sister cohesion during metaphase I-to-anaphase I transition. Sgo1 may also execute its protective role in a PP2A-independent way, because the cohesin protection mediated by the enforced expression of Sgo1 in mitotic fission yeast cells is not abolished even if endogenous B' subunits, which are essential for PP2A–Sgo1 interaction, are depleted from the cells (Kitajima et al. 2006).

4.2

Protection of Centromeric Cohesion at Mitosis

The protection of centromeric cohesion by the shugoshin–PP2A complex occurs not only in meiosis, but also in mitosis, at least in vertebrates (Fig. 4). Human cells have two shugoshin family proteins, hSgo1 and hSgo2, and both are expressed during mitosis. During prophase in proliferating cells, cohesins are removed from the chromosome arms but not from the centromeres by the

prophase pathway, which does not involve cleavage of the Scc1/Rad21 subunit by separase. Phosphorylation of the SA2 subunit (Scc3 homolog) of cohesin by a Polo-like kinase 1 (Plk1) is crucial for this regulation because the depletion of Plk1 or the expression of a non-phosphorylatable mutant of SA2 blocks dissociation of cohesin from all chromosomal regions at prophase and prometaphase (Hauf et al. 2005; Losada and Hirano 2002; Sumara et al. 2002). The depletion of hSgo1 in proliferating cells causes the removal of cohesins even from centromeres by a prophase pathway (Kitajima et al. 2005; McGuinness et al. 2005; Tang et al. 2004). Consequently, sister chromatids separate precociously before metaphase. It is speculated that a mechanism protecting cohesin at centromeres by hSgo1 might be the inhibition of SA2 phosphorylation since the dissociation of cohesin from centromeres and the premature separation of sister chromatids observed in hSgo1-depleted cells are rescued by the expression of a non-phosphorylatable SA2 (McGuinness et al. 2005).

The Sgo1–PP2A interaction is also conserved in mammals (Kitajima et al. 2006; Riedel et al. 2006; Tang et al. 2006). PP2A associates and colocalizes with shugoshin at centromeres, and is required for centromeric protection. Importantly, the purified hSgo1–PP2A complex has the ability to reverse the phosphorylation state of the SA2 subunit *in vitro*, suggesting that the dephosphorylation of cohesin is the mechanism by which centromeres are protected (Kitajima et al. 2006). In spite of such a protective role, hSgo1 apparently does not protect cohesin from separase cleavage at the onset of anaphase. Consistently, HeLa cells expressing a non-phosphorylatable SA2 undergo anaphase and their chromosomes separate normally although cohesin along the chromosome arms is not removed by the prophase pathway, indicating that at least the phosphorylation of SA2 is not an essential modification to promote the cleavage of Scc1 by separase (Hauf et al. 2005). The knockdown of hSgo2 causes a modest defect in chromosome alignment and centromeric protection of cohesin (Kitajima et al. 2006). Therefore, although the precise role of hSgo2 remains unclear, the roles of hSgo1 and hSgo2 are diverged in human cells. The meiotic roles of shugoshin in higher eukaryotes remain to be examined.

4.3

Another Role of Shugoshin

Whereas fission yeast Sgo1 is meiosis-specific, Sgo2 is ubiquitously expressed throughout the mitotic as well as the meiotic cell cycle. Sgo2 localizes at the pericentromeric heterochromatin regions at metaphase, but it is dispensable for the centromeric protection of Rec8-cohesin at meiosis I. Instead, the deletion of Sgo2 results in a modest defect in the fidelity of chromosome segregation in mitosis and homolog disjunction in meiosis I, indicating that Sgo2 plays an important role in general chromosome segregation (Kitajima et al. 2004; Rabitsch et al. 2004). Crucially, Sgo1 in budding yeast was iden-

tified as a component that senses microtubule tension at kinetochores and activates the mitotic spindle assembly checkpoint when spindle tension is perturbed (Indjeian et al. 2005). This indicates that budding yeast Sgo1, which is a unique ortholog of shugoshin family proteins in this organism, is required not only to protect centromeric Rec8 at meiosis I, but also to sense the lack of tension on kinetochores. In *Xenopus*, Sgo1 was identified as a factor that promotes microtubule polymerization in vitro (Salic et al. 2004), suggesting the possibility that shugoshin interacts with microtubules at centromeres, thereby sensing tension (Indjeian et al. 2005). Interestingly, however, recent analysis has revealed that fission yeast Sgo2 also regulates tension-generating kinetochore attachment by recruiting the chromosomal passenger complex (CPC), an essential component acting in the tension-sensing spindle assembly checkpoint pathway, to centromeres in both mitosis and meiosis (Kawashima et al. 2007).

Whereas fission yeast as well as plants and vertebrates possess two shugoshin family proteins, budding yeast and *Drosophila* have only one (Watanabe 2005). Thus, in fission yeast, the original functions of shugoshin might have been split into Sgo1 and Sgo2 during evolution, with the former exclusively responsible for the centromeric protection of Rec8 at meiosis I, and the latter responsible for activating the tension-sensing spindle assembly checkpoint. The functional difference between vertebrate Sgo1 and Sgo2, and whether at least one of them has a role at the tension-sensing spindle assembly checkpoint remains to be addressed.

4.4

Regulation of Shugoshin Function

In fission yeast, a key upstream regulator of the centromeric localization of shugoshin is a conserved kinase, Bub1, which is essential for the spindle assembly checkpoint. In the Bub1-depleted cells, the centromeric localization of both Sgo1 and Sgo2 is abolished (Kitajima et al. 2004). Consistently, centromeric Rec8 fails to persist during meiosis I in *bub1* mutant cells (Bernard et al. 2001a). Thus, the Bub1-regulated spindle assembly checkpoint could be partly, if not entirely, attributable to Sgo2–CPC function. Remarkably, the centromeric localization of budding yeast Sgo1 or human shugoshins also depends on Bub1 function, indicating that the fundamental relationship of shugoshin–Bub1 is evolutionally conserved (Kitajima et al. 2005; Riedel et al. 2006; Tang et al. 2004; Kiburz et al. 2005). In Bub1-depleted HeLa cells, Sgo1 is not only displaced from the centromeres but also is found at low levels along the entire chromosome length, resulting in the ectopic protection of cohesin from the prophase pathway along the entire chromosomal region. This indicates that Sgo1 can associate with chromosomes and preserves the function of protecting cohesin, but cannot localize specifically at the centromeres in the absence of Bub1. Thus, the function of Bub1 seems to be essential to restrict

the association of Sgo1 (and likely PP2A) with chromatin to centromeres. Similar results were obtained in the budding yeast *bub1* mutant (Riedel et al. 2006). How Bub1 kinase regulates the centromeric localization of shugoshin is not yet clear.

In *Drosophila*, both CPC and a Polo-like kinase, Polo, interact directly with and phosphorylate shugoshin Mei-S332. The CPC-dependent phosphorylation promotes the centromeric localization of Mei-S332, whereas the Polo kinase-dependent phosphorylation seems to promote the dissociation of Mei-S332 from centromeres at metaphase-to-anaphase transition (Clarke et al. 2005; Resnick et al. 2006). In *Xenopus* fission yeast and budding yeast, the Sgo1 protein is degraded in an APC/c-dependent manner after the metaphase-to-anaphase transition, whereas fission yeast Sgo2 and *Drosophila* Mei-S332 are dissociated from the centromeres but are not degraded during anaphase (Kitajima et al. 2004; Lee et al. 2005; Marston et al. 2004; Salic et al. 2004). Therefore, the mechanisms of the inactivation of shugoshin family members are somewhat diverged.

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Spo11 and the Formation of DNA Double-Strand Breaks in Meiosis

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Abstract Meiotic recombination is carried out through a specialized pathway for the formation and repair of DNA double-strand breaks made by the Spo11 protein, a relative of archaeal topoisomerase VI. This review summarizes recent studies that provide insight to the mechanism of DNA cleavage by Spo11, functional interactions of Spo11 with other proteins required for break formation, mechanisms that control the timing of recombination initiation, and evolutionary conservation and divergence of these processes.

Abbreviations

DSB	DNA double-strand break
CAP	catabolite activating protein
CDK	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
MRX	Mre11-Rad50-Xrs2 complex
TopoVI	topoisomerase VI

1

Double-Strand Breaks and the Initiation of Meiotic Recombination

Most sexually reproducing organisms use recombination to connect homologous paternal and maternal chromosomes to one another during prophase I of meiosis (Page and Hawley 2003; Petronczki et al. 2003). This connection is essential for accurate chromosome segregation at the first meiotic division. – Notable exceptions to this pattern are dipteran males (e.g., *Drosophila melanogaster*) and lepidopteran females (e.g., the silk moth *Bombyx mori*), in which meiotic segregation of homologous chromosomes is achieved without recombination (Rasmussen 1977; McKee 1998; McKim et al. 1998). In both classes of insect, however, the other sex does utilize meiotic recombination.

Meiotic recombination has at its heart the formation and subsequent repair of DNA double-strand breaks (DSBs) (reviewed in Keeney 2001). The major steps along the recombination pathway have been best defined in the budding yeast *Saccharomyces cerevisiae* (Fig. 1) (Bishop and Zickler 2004;

Hunter 2007). DSB formation is catalyzed by Spo11, which appears to act via a topoisomerase-like reaction to generate a transient, covalent protein-DNA intermediate (de Massy et al. 1995; Keeney and Kleckner 1995; Liu et al. 1995; Bergerat et al. 1997; Keeney et al. 1997). After DSBs are formed, Spo11 is removed from the DNA (Neale et al. 2005) and the 5' strand termini are nucleolytically resected to yield variable-length, 3' single-stranded tails (Sun et al. 1991; Bishop et al. 1992). In a series of reactions dependent on yeast homologs of bacterial RecA (Bishop et al. 1992; Shinohara et al. 1992), these tails undergo strand invasion of intact homologous duplexes, ultimately giving rise to mature recombinant products (Smith and Nicolas 1998; Paques and Haber 1999; Bishop and Zickler 2004). The repair of any given meiotic DSB can result in either reciprocal exchange of the chromosome arms flanking the break (a crossover), or no exchange of flanking arms (a noncrossover or parental configuration).

Ample evidence indicates that DSBs are initiators of meiotic recombination in other organisms as well. Meiosis-specific DSBs have been demonstrated in *Schizosaccharomyces pombe*, dependent on genes known to be required for meiotic recombination, including the *SPO11* homolog *rec12⁺* (Cervantes et al. 2000). Meiotic DNA strand breaks have also been detected by PCR or in situ DNA labeling assays in mouse spermatocytes (Zenvirth et al. 2003; Qin et al. 2004). Although DSBs have not been directly detected by physical assays in other organisms, Spo11 orthologs are present and are required

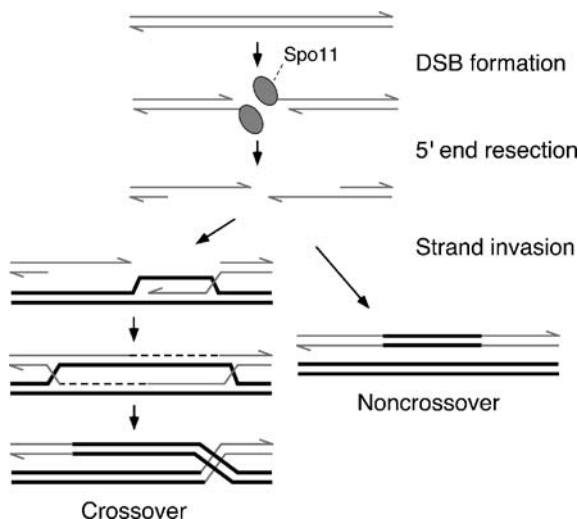


Fig. 1 Overview of meiotic recombination pathway in *S. cerevisiae*. Schematic figures of the DNA at different steps (precursor, intermediates, and products) are shown. Proteins are omitted except for Spo11, which becomes covalently attached to the DNA as part of the DNA cleavage reaction. Only one of the two sister chromatids from each homolog is shown. See text for details

for initiation of meiotic recombination (detected genetically or cytologically) in all sexually reproducing species tested (discussed further in Sect. 2). In many organisms, the recombination defect in *spo11* mutants can be at least partially rescued by production of DSBs from an exogenous source such as ionizing radiation (Thorne and Byers 1993; Dernburg et al. 1998; Celerin et al. 2000; Liu et al. 2002; Storlazzi et al. 2003). Finally, γ H2AX (a phosphorylated form of histone H2AX that is thought to be a marker for the location of DSBs) appears on meiotic chromosomes in many organisms from leptotema through zygonema in a *Spo11*-dependent fashion (reviewed in Fernandez-Capetillo et al. 2004). Thus, DSB formation appears to be a universal feature of meiotic recombination.

There are a number of important questions concerning the initiation of meiotic recombination. For example, what are the biochemical mechanisms of DSB formation and the early steps in processing DSB ends to prepare them for subsequent strand exchange reactions? What are the functions of the many proteins that collaborate with Spo11 to promote DSB formation? How is Spo11 activity controlled so that it functions only at the right time and place? How are specific sites chosen for DSB formation?

This review will focus on relatively recent findings that address these questions. Much of our understanding of these processes derives from studies of *S. cerevisiae*, but increasing detail is emerging from studies of other organisms as well. Similarities and differences between organisms are informative with respect to evolutionary conservation of the mechanism and regulation of meiotic recombination initiation.

2

Spo11 and Its Relation to Archaeal Topoisomerase VI

2.1

Topoisomerase VI

Because Spo11 is related to DNA topoisomerases, a consideration of how topoisomerases work is useful for understanding the mechanism of Spo11 action during meiosis. Topoisomerases catalyze DNA strand breakage and reunion reactions in order to solve topological problems inherent to chromosome metabolism. Various topoisomerases can relax supercoiled DNA, supercoil relaxed DNA, and/or link and unlink intertwined single stranded or duplex DNA molecules (reviewed in Champoux 2001; Wang 2002; Corbett and Berger 2004). These topological changes are carried out through a series of transesterification reactions in which a tyrosine residue on the enzyme attacks a phosphorus in the DNA, forming a tyrosyl phosphodiester link to the DNA and simultaneously severing the DNA backbone (Fig. 2). The resulting DNA break provides a gate through which another DNA strand or duplex can

pass. The DNA backbone is subsequently resealed through the reverse of the cleavage reaction. Topoisomerases are broadly grouped into two main categories: type I enzymes cleave a single strand, whereas type II enzymes cleave both strands of a duplex in concert. Topoisomerases can also be distinguished based on the polarity of their covalent attachment to DNA: some enzymes generate a 3' attachment, while others (including all type II enzymes) become linked to 5' strand termini (Fig. 2).

Most type II topoisomerases of mammals, yeast, and other eukaryotes belong to a single group (the type IIA family), which also includes gyrase and topoisomerase IV of bacteria (Gadelle et al. 2003; Corbett and Berger 2004). Members of this family are also found in some phages and viruses and in some archaeal species. Type IIA topoisomerases share substantial protein sequence and structural homology with one another. They are modular enzymes with subdomains that carry out distinct functions: binding and cleaving DNA, binding and hydrolyzing ATP, and transducing ATP-dependent

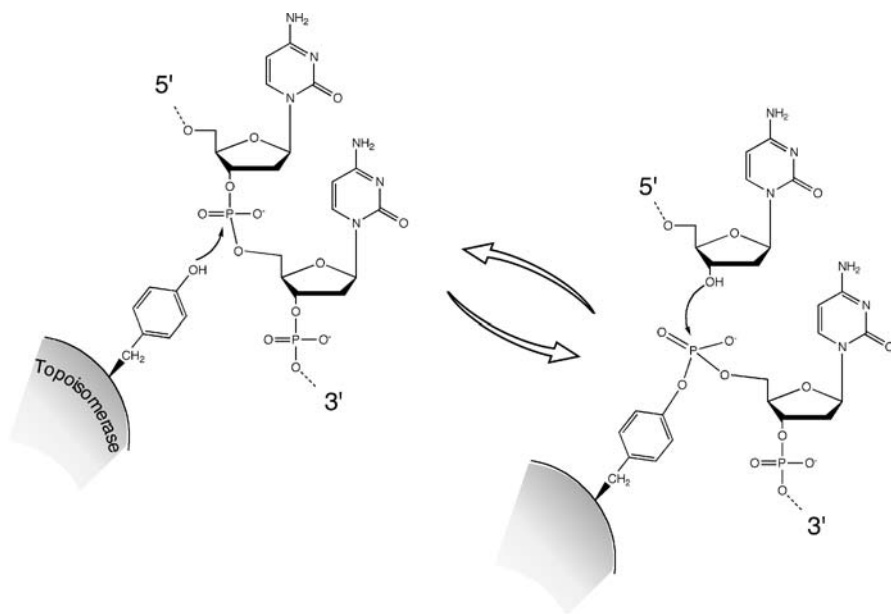


Fig. 2 Chemistry of the DNA cleavage and religation reactions catalyzed by topoisomerases. A tyrosine side chain on the topoisomerase protein carries out a nucleophilic attack on the DNA phosphodiester backbone. This transesterase reaction severs the DNA backbone and covalently links the protein to the DNA end via a tyrosyl phosphodiester linkage. The protein-DNA linkage is reversed and the DNA is resealed by attack of the deoxyribose hydroxyl. In this example, the tyrosyl phosphodiester links the protein to the 5' end of the cleaved strand, as is seen in some type I and all type II topoisomerases. For a type II enzyme, two topoisomerase monomers work in concert to cleave the two strands of the duplex

conformational signals between domains (Corbett and Berger 2004). The catalytic tyrosine residue is carried in an alpha-helical domain similar to the DNA binding domain of catabolite activating protein (CAP) (Berger et al. 1998); additional active site residues are provided in trans from the dimer partner in the form of a divalent metal ion binding pocket formed by a module called a Toprim domain (Aravind et al. 1998; Berger et al. 1998). ATP binding and hydrolysis are carried out by a domain related to ATPase domains of heat shock protein 90, the CheA histidine kinase, and the MutL component of DNA mismatch repair systems (Dutta and Inouye 2000).

The major archaeal type II topoisomerase (called topoisomerase VI) is structurally very different, and is thus in a separate family, called type IIB (Bergerat et al. 1997; Nichols et al. 1999). TopoVI has the same structural and catalytic modules as type IIA enzymes, but the sequence conservation between TopoVI and type IIA enzymes within the modules is limited (as low as $\sim 10\%$ identity) and the order of the modules in the polypeptide sequence and their three-dimensional arrangement differs (Gadelle et al. 2003; Corbett and Berger 2004). TopoVI is an A_2B_2 heterotetramer. The A subunit carries the DNA cleaving catalytic tyrosine and the Toprim metal-binding domain. The B subunit carries the ATP binding domain and a domain thought to be involved in transducing conformational signals between the subunits. Direct enzymatic analysis showed that TopoVI cleaves DNA to yield a two-

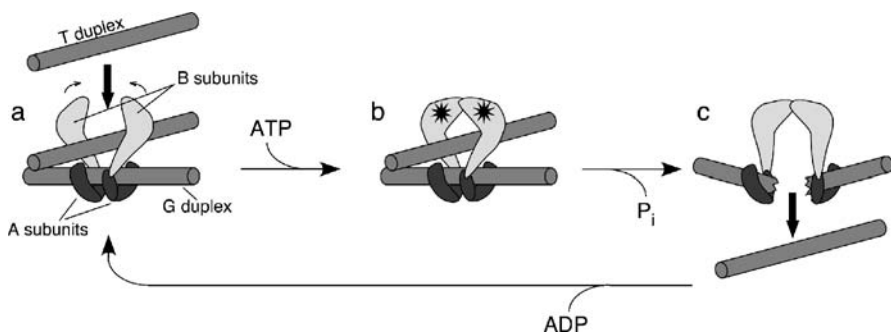


Fig. 3 Structural model for the catalytic cycle of topoisomerase VI. A two-gate model for TopoVI action has been proposed based on enzymatic and structural studies (Corbett and Berger 2004). Two B subunits are proposed to bind to a dimer of A subunits. The subunit-B binding interface on A is thought to reside on the CAP domain, which also contains the catalytic tyrosine residue. The dimer of A subunits binds to the DNA duplex that will be cleaved (the G, or gate, duplex). A second DNA duplex (the T, or transfer, duplex) enters the enzyme through a gate between the ATPase domains of the B subunits (*panel a*). ATP binding (*stars*) induces dimerization of the B subunits, trapping the T duplex (*panel b*). A conformational change accompanying ATP hydrolysis and DNA cleavage opens a gate in the G duplex, through which the T duplex is expelled (*panel c*). Release of ADP and resealing of the G duplex recycle the enzyme to its starting state

nucleotide 5' overhang and that cleavage by the A subunit is absolutely dependent on both the B subunit and ATP (Buhler et al. 1998, 2001).

Structures are available for fragments of TopoVI A and B subunits (Nichols et al. 1999; Corbett and Berger 2003; Corbett and Berger 2005). On the basis of these, Berger and colleagues have proposed a rough structural model for the holoenzyme as follows: Two B subunits sit atop a dimer of A subunits that themselves bind to the DNA that will be broken (the gate, or "G" duplex) (Corbett and Berger 2003, 2004) (Fig. 3a). The B subunits are thought to contact the CAP domains of the A subunits and thus to influence directly the position of the catalytic tyrosine relative to DNA and to the metal binding pocket in the Toprim domain. Upon ATP binding, the B subunits dimerize (Corbett and Berger 2003) (Fig. 3b), trapping a second DNA duplex (the transfer, or "T" duplex) and activating cleavage of the G duplex. ATP hydrolysis then drives a conformational change that disrupts the A subunit dimer interface, breaking the G duplex and opening a gate through which the T duplex is expelled (Fig. 3c). Release of ADP allows the enzyme to recycle to the starting state. In its broad outlines, this ATP-driven gating model for strand passage for TopoVI is analogous to that proposed for the type IIA enzymes (Roca et al. 1996).

2.2

Spo11

The discovery that meiotic DSBs are made by a topoisomerase relative grew from studies in *S. cerevisiae* a little over a decade ago. Early observations established that meiotic DSBs have protein covalently attached to the 5' DNA strand termini on both sides of the break in mutants (*rad50S*, *mre11S*, and *sae2Δ*) that accumulate unrepaired DSBs that are not exonucleolytically resected (de Massy et al. 1995; Keeney and Kleckner 1995; Liu et al. 1995). This finding suggested that DSBs are generated by a topoisomerase-like transesterification reaction involving a covalent protein-DNA intermediate. With this knowledge as the backdrop, the role of Spo11 as the catalytic subunit for meiotic DSB formation was uncovered through two separate lines of inquiry. In one, sequencing of the genes for the first archaeal TopoVI revealed homology between the TopoVIA subunit and yeast Spo11 (Bergerat et al. 1997). The significance of this homology with respect to the biochemical function of Spo11 was immediately recognized and confirmed by genetic tests in *S. cerevisiae* (Bergerat et al. 1997). Separately, covalent protein-DSB complexes were purified from meiotic *rad50S* cells and the protein component was directly shown to be Spo11 (Keeney et al. 1997).

Earlier genetic studies of *SPO11* have been reviewed in detail (Keeney 2001). More recently, mutational analyses of *S. cerevisiae* Spo11 and the *S. pombe* ortholog Rec12 have confirmed the biochemical and structural similarity of Spo11 to the TopoVIA subunit (Diaz et al. 2002; Sharif et al. 2002;

DeWall et al. 2005; Nag et al. 2006). In particular, residues have been identified in Spo11 and Rec12 that are equivalent to the catalytic tyrosine and acidic residues that coordinate a divalent metal ion in archaeal TopoVI. Also, TopoVIA is dimeric and forms hybrid active sites for DNA cleavage; each active site involves the CAP domain of one monomer and the Toprim domain of the other (Nichols et al. 1999). The semidominant phenotypes of *S. cerevisiae* *spo11* mutants that lack key catalytic residues are consistent with the prediction that Spo11 has the same three-dimensional architecture (Diaz et al. 2002). When Spo11 proteins with different affinity tags are coexpressed, they can be coimmunoprecipitated, also consistent with formation of dimers or higher order oligomers (Sasanuma et al. 2007). Moreover, like TopoVI, Spo11 cleaves DNA to yield a two-nucleotide 5' overhang (de Massy et al. 1995; Liu et al. 1995). The evidence in support of Spo11 as the catalyst for DSB formation is thus very strong. Direct biochemical demonstration of this activity in vitro has thus far proved elusive, however.

It is likely that an ancestral form of TopoVI evolved prior to the divergence of eukaryotic and archaeal lineages, and that with the evolution of meiosis and meiotic recombination the A subunit was subsequently adapted to perform a new function (Gadelle et al. 2003; Corbett and Berger 2004). Interestingly, there is no obvious equivalent of the B subunit of TopoVI in most eukaryotes, aside from some plants. On the basis of enzymatic and structural studies, the TopoVIB subunit is known or inferred to have several functions, some of which are dependent on binding and/or hydrolysis of ATP (Corbett and Berger 2003, 2004, 2005): (1) activating DNA cleavage by TopoVIA, presumably by influencing the position and orientation of the structural domain that contains the catalytic tyrosine; (2) inducing a conformational change that forces apart the TopoVIA subunits upon DNA cleavage; (3) providing a bridge that holds the two ends of the broken DNA together within the holoenzyme-DNA complex until the DNA duplex is resealed (Fig. 3). Although there is no reason to suspect that Spo11 carries out a strand passage reaction, Spo11 may perform at least a subset of the same or analogous catalytic steps and conformational changes during meiotic DSB formation as are performed by the TopoVIA subunit (discussed further in Sect. 5). Thus, although it is possible that the functions performed by the TopoVIB subunit are not required for Spo11, it is also possible that some other protein(s) has been recruited to functionally replace the B subunit. One or more of the proteins in addition to Spo11 that are known to be required for DSB formation are excellent candidates (discussed in Sect. 3). If so, however, these proteins could presumably replace only ATP-independent functions of the B subunit, since none of these known proteins (except Rad50) has an ATP-binding motif.

Spo11 is highly conserved. The function of Spo11 orthologs in initiating meiotic recombination has been demonstrated in other fungi and in nematode, *Drosophila*, and mouse (Dernburg et al. 1998; McKim and Hayashi-Hagihara 1998; Baudat et al. 2000; Celerin et al. 2000; Romanienko and

Camerini-Otero 2000; Steiner et al. 2002; Storlazzi et al. 2003; Bowring et al. 2006). Spo11 homologs have also been identified in plants, but in a genetically more complex arrangement than in many other organisms. Specifically, the *A. thaliana* genome encodes three genes homologous to *SPO11*, called *AtSPO11-1*, *AtSPO11-2*, and *AtSPO11-3* (Hartung and Puchta 2000, 2001; Grelon et al. 2001). Interestingly, both *AtSPO11-1* and *AtSPO11-2* are essential for initiation of meiotic recombination (Grelon et al. 2001; Stacey et al. 2006). How the products of these two genes work together to promote recombination is not yet clear, but it is tempting to speculate that DSBs in this organism are made by an obligate heterodimer of different Spo11-related subunits. *AtSPO11-3* is dispensable for meiotic recombination, and instead encodes a protein that interacts with a homolog of TopoVIB to constitute a bona fide TopoVI relative (Hartung et al. 2002; Sugimoto-Shirasu et al. 2002, 2005; Yin et al. 2002).

Spo11 appears to be present in all sequenced eukaryotic genomes, and indeed it may be the only truly universal meiotic protein (Ramesh et al. 2005) (S.-B. Malik and J. Logsdon, personal communication). Interestingly, Spo11 homologs are present even in lineages that are thought to be asexual and/or to not have a meiotic phase in their life cycles (Ramesh et al. 2005; Richard et al. 2005). The presence of Spo11 in the genome may signal the operation of cryptic sexuality in these species, not enough evolutionary time to lose the gene since the evolution of asexuality in the lineage, and/or retention of Spo11 because it has another function(s) aside from inducing meiotic recombination. Given the precedent of *AtSPO11-3*, it is also possible that Spo11 homologs in some eukaryotes are TopoVI subunits rather than Spo11 orthologs, although thus far clear orthologs of the TopoVIB subunit have only been identified in higher plants.

In several fungi and in *C. elegans*, Spo11 expression is limited primarily or exclusively to meiotic cells (Atcheson et al. 1987; Lin and Smith 1994; Dernburg et al. 1998; Celerin et al. 2000; Storlazzi et al. 2003). However, in other organisms *Spo11* message has been detected in nonmeiotic cells, although no function for the gene has been demonstrated in these cases. In one example, *Spo11* message was detected in mouse germinal center B cells undergoing immunoglobulin gene diversification and class switch recombination, but mice lacking *Spo11* had no detectable immune system defects (Klein et al. 2002). Another example is *Drosophila*, in which the *Spo11* homolog *meiW-68* is expressed in testis (where no meiotic recombination occurs) and in somatic cells of embryos and pupae (McKim and Hayashi-Hagihara 1998; Lankenau 2006).

The association of Spo11 with meiotic chromosomes has been analyzed in several organisms. Cytological studies showed Spo11 forming discrete staining structures ("foci") on chromatin as early as leptotema in mouse, *Sordaria macrospora*, and *S. cerevisiae* (Romanienko and Camerini-Otero 2000; Storlazzi et al. 2003; Prieler et al. 2005). This is the period when DSBs are formed,

so these cytologically visible Spo11 complexes are present at the right time to be involved in DSB formation. Somewhat surprisingly, however, Spo11 persists on chromosomes into the pachytene stage in each of these organisms, past the time of DSB generation. As discussed below, similar behavior is seen for the other yeast DSB proteins; it is not yet known whether this persistent association with chromosomes has a function, and if so, what that function might be.

Chromatin immunoprecipitation (ChIP) has provided an even more detailed picture of Spo11 binding to chromatin in *S. cerevisiae*, and has demonstrated a preferential association of Spo11 with at least some DNA sequences where DSBs frequently form (i.e., DSB hotspots) (Prieler et al. 2005). The ChIP study by Klein and colleagues revealed a particularly intriguing aspect of Spo11 binding to chromatin (Prieler et al. 2005). In *rad50S* mutants at relatively late time points, covalent association of Spo11 with hotspots could be detected without the need to crosslink with formaldehyde, as expected since Spo11 remains covalently bound to DSB ends in this mutant. Surprisingly, however, Spo11 binding to hotspots could also be detected without crosslinking at earlier time points, prior to the formation of DSBs. This “tight” binding could be disrupted with high salt, and the strands of the Spo11-associated DNA were intact, so Spo11 was not covalently linked to the DNA in these complexes. Importantly, these complexes were not seen in wild-type cells, and they formed in the *rad50S* mutants at the same time that DSBs would have appeared in wild type (the *rad50S* DSBs were slightly delayed)¹. Prieler et al. have proposed that these “tight” Spo11-DNA complexes are an intermediate in the normal DSB pathway in which Spo11 has been activated in some way. Their findings may have important implications for understanding the mechanism of DSB formation as well as for understanding the nature of the defect in *rad50S* mutants. These issues are discussed further in Sect. 5.

2.3

Formation and Early Processing of Spo11-Dependent DSBs

Extrapolating from the structure of the TopoVIA subunit provides a detailed picture of what Spo11 might look like as it cleaves DNA (Fig. 4). Binding of a Spo11 dimer to DNA would position the metal binding pocket of each monomer close to the scissile bond on one of the strands of the duplex (Fig. 4a). The catalytic tyrosine residue of one monomer (Tyr-135 in *S. cerevisiae* Spo11) interacts with the metal binding pocket on the other monomer.

¹ It has also been demonstrated that DSBs in *rad50S* and *sae2* mutants have a different broad-scale distribution than DSBs in wild-type cells (Dresser et al. 1997; Borde et al. 2000). In particular, DSBs that normally form in late-replicating portions of chromosomes (e.g., near telomeres) are specifically reduced or absent in *rad50S* and *sae2* mutants (Borde et al. 2000). These findings provide further evidence that *rad50S* DSBs are not simply “normal” breaks that cannot be repaired.

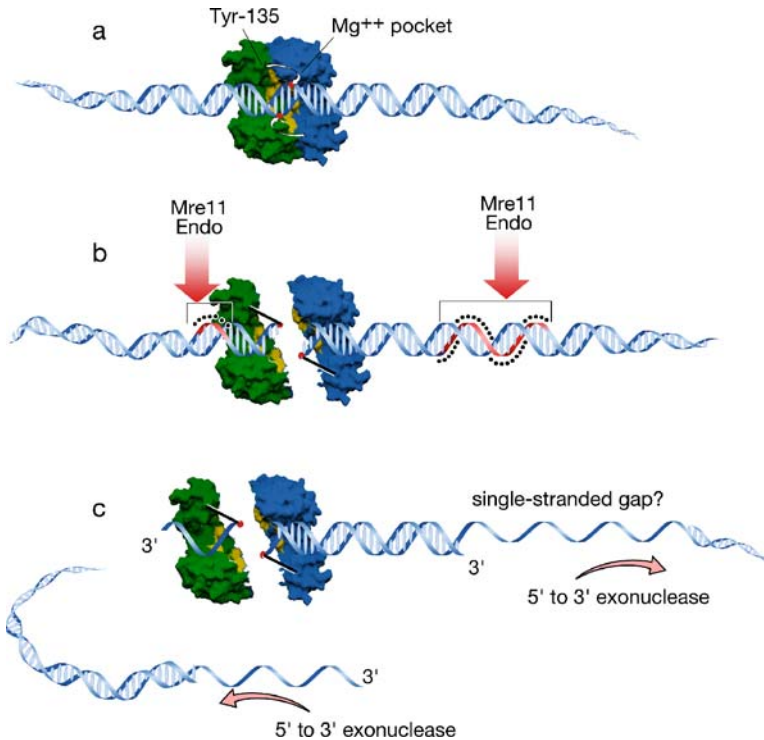


Fig. 4 Structural model for Spo11-induced DSB formation **a** A Spo11 dimer is modeled using a surface rendering of the structure of *Methanococcus jannaschi* TopoVIA, with one monomer in green and the other in blue (Nichols et al. 1999). Approximate positions of the catalytic tyrosine of one monomer and the metal-binding pocket of the other monomer are indicated. B-form DNA (drawn approximately to scale) is docked onto the dimer. The metal binding pockets of the two monomers are appropriately spaced to place them in proximity to the scissile phosphates which would give the two-nucleotide 5' overhang known to be generated by Spo11 and TopoVI. The tyrosines in the TopoVIA structure are far from these phosphates, however, so it is assumed that a conformational change in the protein moves the tyrosines into position to cleave (Nichols et al. 1999) (small white arrows). **b** Cleavage of the DNA backbone leaves Spo11 covalently attached to the DSB ends. For clarity, the Spo11 dimer interface has been interrupted to show how the two monomers are attached. Analogous separation of the DNA ends is what opens a gate to allow strand passage for TopoVI (Corbett and Berger 2004); whether the Spo11 dimer interface might be disrupted in this manner is not known (see Sect. 5). After DNA cleavage, Spo11 is released from DSB ends by endonucleolytic single-strand cleavage on either side of the break, most likely mediated by Mre11 endonuclease activity. The strands where nicking occurs are colored red and highlighted with the black dots. Asymmetric nick spacing is shown, but the disposition of nicks around individual DSBs is not yet known. **c** The DSB ends are further processed by 5' → 3' single-strand resection. The shorter Spo11-bound oligonucleotides may be small enough to be readily dissociated, but the longer oligonucleotides would need to be actively unwound. This feature may result in capping of one of the DSB ends, which may in turn influence the behavior of this end during subsequent recombination reactions (Neale et al. 2005)

Two hybrid active sites are thus formed on either side of the duplex². Nucleophilic attack by the tyrosine residues cleaves the DNA strands and generates a DSB with Spo11 covalently attached to the 5' strand termini. If the dimer interface were to be disrupted at this point (as cartooned in Fig. 4b), the ends of the DSB would also be separated as in the strand passage gate opened in TopoVI (Fig. 3).

Recent work in *S. cerevisiae* and mouse has demonstrated that Spo11 is removed from DSB ends by an endonucleolytic mechanism in which nicks are introduced into the Spo11-bound strands adjacent to the DSB (Fig. 4b) (Neale et al. 2005). This reaction releases Spo11 still covalently bound to a short oligonucleotide. Presumably, the nicks serve as starting points for further 5' → 3' exonucleolytic processing. The endonuclease activity of Mre11 is an excellent candidate for clipping off Spo11 (Sect. 3.1). The exonuclease responsible for more extensive end resection is not yet known, although Exo1 may play a role (reviewed in Hunter 2007).

Spo11-associated oligonucleotides are heterogeneous in length and in *S. cerevisiae* they fall into two discrete size classes of roughly equal abundance: about half of the oligonucleotides are ~ 7–12 nucleotides in length, and the remainder are ~ 21–37 nucleotides (Neale et al. 2005). How these two populations arise is not yet known, but they have been proposed to result from asymmetric spacing of the nicks on either side of the DSB. In any case, the shorter oligonucleotides would be derived from cleavage at the edge of the protein-DNA footprint predicted from the TopoVIA structure, whereas the longer oligonucleotides would result from cleavage several turns of the helix away (Fig. 4b). Because of the two-nucleotide 5' overhang of the DSB, the shorter oligonucleotides would only form 5–10 bp of duplex which might be readily disrupted *in vivo*. In contrast, the longer oligonucleotides would need to be actively unwound from the 3'-terminal strand. These considerations have suggested a model in which one side of the DSB ends in a free 3' strand whereas the other side is capped because it remains base-paired to the Spo11-associated oligonucleotide (Fig. 4c) (Neale et al. 2005). Such an asymmetric structure might dictate the asymmetric behavior of the two ends of the DSB in which one end invades a homologous duplex and the other DSB end is captured in a second reaction (Fig. 1).

Unless the covalently bound oligonucleotide can be removed, Spo11 would be irreversibly consumed when it makes a DSB. It has been speculated that this suicide mechanism might be a means of limiting the numbers of DSBs (Keeney et al. 1997), but in fact there is considerably more Spo11 protein in the cell than what catalyzes DSB formation (Neale et al. 2005). Moreover, Spo11 persists on chromatin and at hotspots well past the time of DSB formation (Romanienko and Camerini-Otero 2000; Storlazzi et al. 2003; Prieler et al.

² Whether Spo11 dimerizes on the DNA or is dimeric in solution, as is TopoVIA (Nichols et al. 1999), remains to be determined (see Sasanuma et al. 2007).

2005). These observations raise the question of what, if anything, the “extra” Spo11 does: is there a function other than making DSBs? More importantly, these results indicate that binding of Spo11 to DNA is not sufficient to make a DSB, but rather that Spo11 must be activated in some way (Prieler et al. 2005). Subsequent sections will discuss some of the ways that this activation might occur and how it is controlled.

3

Other Proteins Required for Meiotic DSB Formation

3.1

DSB Proteins in *S. cerevisiae*

Spo11 is the catalytic center of the meiotic recombination initiation mechanism, but it is not sufficient to generate DSBs: numerous additional proteins are also required. In *S. cerevisiae*, at least nine other proteins are essential for Spo11 activity. Increasing detail is emerging about the interactions among these proteins, but their precise functions are not yet well understood.³

Ski8. Ski8 (also known as Rec103) is particularly intriguing because it has two very distinct physiological roles: during meiosis, it is required for DSB formation (Gardiner et al. 1997; Tessé et al. 2003), but in vegetative cells it is involved in RNA metabolism. Ski8 forms a cytoplasmic complex with Ski2 and Ski3 (Brown et al. 2000; Wang et al. 2005) that is required for inhibition of translation of nonpolyadenylated RNA and for 3′ → 5′ exonucleolytic degradation of RNA (Masison et al. 1995; Jacobs Anderson and Parker 1998; van Hoof et al. 2000; Araki et al. 2001). During meiosis, however, Ski8 relocates from the cytoplasm to the nucleus and associates with meiotic chromosomes, dependent on Spo11 in both *S. cerevisiae* and *S. macrospora* (Tessé et al. 2003; Arora et al. 2004). *S. cerevisiae* Ski8 interacts with Spo11 in two-hybrid assays, and point mutations in Spo11 that disrupt this interaction also eliminate DSB formation (Arora et al. 2004). It thus appears that Ski8 is a direct partner of Spo11 and that interaction between the two proteins is essential for DSB formation. Ski8 is required for association of Spo11 with chromosomes in *S. macrospora* (Tessé et al. 2003). The same is not true in *S. cerevisiae* (Prieler et al. 2005), but bulk chromatin fractionation assays indicate that binding of Spo11 to chromatin is at least partially reduced in the absence of Ski8 (Arora et al. 2004). A Ski8 ortholog, Rec14, is required for meiotic DSBs in *S. pombe* (Evans et al. 1997; Fox and Smith 1998; Davis and Smith 2001; Molnar et al.

³ This section focuses on the subset of proteins thought to be most directly involved in promoting Spo11-dependent DSB formation. Not considered here are numerous other factors that are required for DSBs, but which are known or suspected to be more indirectly involved. These include regulatory factors that control the decision to enter meiosis and transcription factors that control expression of Spo11 or other meiotic recombination proteins.

2003), but the role of Ski8 in promoting Spo11 activity does not appear to be universal since the *Arabidopsis* homolog is not required for meiotic recombination (Jolivet et al. 2006).

Ski8 is a member of the WD repeat family of proteins, and crystallographic studies revealed that Ski8 has the toroidal β -propeller structure characteristic of this family (Cheng et al. 2004; Madrona and Wilson 2004). It is straightforward to think that, like other known WD proteins, Ski8 bridges interactions between other proteins. Studies in *S. cerevisiae* are consistent with this hypothesis: Ski8 is required in order for Spo11 to interact with Rec104 in two-hybrid assays (Arora et al. 2004). However, this begs the question, why are these bridging interactions important for Spo11 to cleave DNA? Moreover, it is not yet known whether Ski8 does something during meiosis in addition to bringing together Spo11 and other proteins.

Mre11-Rad50-Xrs2 (MRX). These proteins form an evolutionarily conserved complex with multiple roles in many different aspects of DNA metabolism, including DNA repair, telomere maintenance, and checkpoint signaling. Mre11 has single-strand endonuclease and 3' $\rightarrow$ 5' exonuclease activities and Rad50 is an ATP-binding protein structurally related to SMC proteins. The structure and functions of these proteins have been reviewed in detail (Assenmacher and Hopfner 2004; Krogh and Symington 2004; Stracker et al. 2004). I will focus here on their roles in meiosis.

The MRX complex is required for DSB formation in *S. cerevisiae*: a null mutation in any of the genes completely eliminates meiotic recombination (reviewed in Keeney 2001). The complex is also required to process the DSBs once they are formed: specific *rad50* and *mre11* point mutations (generally called *rad50S* and *mre11S* mutations) cause DSBs to accumulate with Spo11 covalently attached to the DNA ends. Deletion of the *SAE2/COM1* gene causes the same phenotype (Keeney and Kleckner 1995; McKee and Kleckner 1997; Prinz et al. 1997). These mutants all share other defects in telomere maintenance and in processing of hairpin DNA ends, consistent with the hypothesis that they share a common biochemical defect (Assenmacher and Hopfner 2004; Krogh and Symington 2004; Stracker et al. 2004). Since *mre11* mutants in this class include mutants known to eliminate the protein's endonuclease activity (Furuse et al. 1998; Moreau et al. 1999), it is reasonable to argue that Mre11 endonuclease is essential for the processing of DSB ends in meiosis, and in particular for endonucleolytic removal of Spo11 from DSB ends (Sect. 2.3) (Neale et al. 2005). However, it is not yet clear why MRX is required for formation of DSBs and not just for DSB processing. One possibility is that the requirement for the presence of MRX facilitates close coordination of DSB formation with subsequent processing of the DSB ends (Borde et al. 2004). Such coordination may help ensure that potentially lethal DSBs are repaired efficiently.

Mre11 is required for both formation and repair of DSBs in *C. elegans*, as in budding yeast (Chin and Villeneuve 2001). In contrast, MRX orthologs

are not required for DSB formation in *S. pombe*, the mushroom *Coprinus cinereus*, or *Arabidopsis*, although they are required for normal repair of meiotic DSBs in all organisms tested (Gerecke and Zolan 2000; Merino et al. 2000; Bleuyard et al. 2004; Puizina et al. 2004; Young et al. 2004). Moreover, in *S. pombe*, a *rad50S*-like mutation and a nuclease-defective allele of *rad32* (the ortholog of *MRE11*) both confer defects in meiotic DSB repair (Farah et al. 2002, 2005b; Steiner et al. 2002; Young et al. 2002). Thus, it appears that the role of MRX in processing DSB ends is evolutionarily conserved, even if the role in promoting DSB formation is not. It has not been possible to address whether members of the complex are required for DSB formation in mouse because null mutations for any of these factors are lethal (Xiao and Weaver 1997; Luo et al. 1999; Zhu et al. 2001). A targeted *Rad50* mutation in mouse (changing Lys-22 to Met, mimicking an *S. cerevisiae rad50S* point mutation) recapitulates the checkpoint hyperactivation phenotype of the yeast mutant, but not the meiotic DSB processing phenotype (Bender et al. 2002; Morales et al. 2005; Usui et al. 2006). However, the corresponding point mutation in yeast (Arg-20 to Met) has a relatively weak phenotype (Alani et al. 1990), and a mimic of a stronger yeast allele does not support growth of mouse embryonic stem cells in culture (Bender et al. 2002), so the question of whether the Mre11 complex processes meiotic DSBs in mammals also remains unanswered.

Several functional subdomains of the MRX proteins important for meiotic recombination have been defined. As mentioned above, the Mre11 nuclease activity is required for Spo11 to be removed from DSB ends. Additionally, the C-terminal region of *S. cerevisiae* Mre11 is required for DSB formation and interacts with as-yet-unknown meiosis-specific proteins (Usui et al. 1998). This domain may not be conserved, however, as mutations that affect the C terminus of mouse MRE11 do not block meiotic DSB formation (Theunissen et al. 2003).

Rad50, as a member of the SMC family, has N- and C-terminal globular domains that come together to form a module for ATP-dependent dimerization and DNA binding (Hopfner 2006). These domains are separated by stretches of heptad repeat sequence that fold back to form a long alpha-helical coiled coil. One end of this coiled coil lies at the ATP binding domain, while the other end has a zinc-hook structure that allows multimerization of MRX complexes (Hopfner et al. 2002). Both ATP binding and zinc-hook driven multimerization are essential for DSB formation (Alani et al. 1990; Wiltzius et al. 2005).

The amino acid sequence of Xrs2 is less well conserved than the other members of the complex, although at least some functional domains are conserved. Xrs2 and its human equivalent NBS1 have N-terminal FHA and tandem BRCT domains (Chahwan et al. 2003; Becker et al. 2006), which are motifs often involved in binding to phosphopeptides (Durocher and Jackson 2002; Glover et al. 2004). *S. cerevisiae* Xrs2 also has conserved C-terminal do-

mains required for interaction with Mre11 and with the kinase Tel1 (Shima et al. 2005; Tsukamoto et al. 2005). The Mre11-interaction domain is required for DSB formation, but the Tel1-interaction domain is not (Shima et al. 2005; Tsukamoto et al. 2005). Xrs2 also interacts with Mer2 in two-hybrid assays, and this interaction requires a serine residue on Mer2 that is phosphorylated by cyclin-dependent kinase (CDK) (see below). It is tempting to speculate that the putative phosphoserine-binding tandem BRCT motif on Xrs2 mediates interaction with phosphorylated Mer2. Indeed, deletion mutations affecting this region in Xrs2 do cause defects in forming and/or repairing DSBs, although this portion of Xrs2 is not essential for these processes (Shima et al. 2005; Tsukamoto et al. 2005).

In genome-wide ChIP analyses, *S. cerevisiae* Mre11 bound preferentially to sites of DSB formation, and association with hotspots required all of the DSB proteins except, surprisingly, Rad50 (Borde et al. 2004). Rad50 was also associated with hotspots by ChIP, but this association required both Mre11 and Xrs2. In *rad50S* or *sae2Δ* mutants, Mre11 ChIP signal persisted at DSB sites in time course experiments, suggesting that the protein remains associated with unresected DSBs (Borde et al. 2004). Persistent accumulation at unresected DSBs could also be detected cytologically as discrete immunostaining foci of MRX proteins on chromosomes (Usui et al. 1998). The specific localization to DSB sites strongly supports the hypothesis that the MRX complex is a direct player in DSB formation and resection.

Rec102-Rec104. The meiosis-specific Rec102 and Rec104 proteins interact genetically and physically in a variety of assays (Salem et al. 1999; Kee and Keeney 2002; Jiao et al. 2003; Kee et al. 2004). Both proteins localize to the nucleus and are associated with meiotic chromosomes starting as early as leptotema, and both continue to accumulate as meiotic prophase progresses before abruptly dissociating from chromatin at mid-pachynema (Kee et al. 2004). They require each other and also Spo11 and Ski8 (but not other DSB proteins) for nuclear localization and for binding to chromatin (Kee et al. 2004). Rec102 and Rec104 are both required for Spo11 to self-associate, to bind to chromatin, to localize to DSB hotspots (Prieler et al. 2005; Sasanuma et al. 2007). ChIP and immunocytological analyses indicated that Rec102 binds preferentially to chromatin loops (as opposed to the chromosome axes), but the protein is associated with both DSB-hot and DSB-cold domains, so its presence cannot be sufficient to specify where DSBs will occur (Kee et al. 2004). Rec104 is phosphorylated during meiosis, and normal phosphorylation patterns require Rec102 but not DSB formation (Kee et al. 2004). The kinase(s) responsible has not been identified, nor is it yet known whether this phosphorylation is required for DSB formation. The close congruence in behaviors of Rec102 and Rec104 strongly suggests that these proteins are a functional unit, and two-hybrid, coimmunoprecipitation, and genetic interactions connect them closely with the Spo11-Ski8 complex (Kee and Keeney 2002; Jiao et al. 2003; Arora et al. 2004). However, they lack identifiable sequence

motifs and the available data do not reveal specific biochemical activities, so precisely what roles they play in DSB formation remains to be determined.

Mer2. The cyclin-dependent kinase Cdc28 and its B-type cyclin partners Clb5 and/or Clb6 are required for early events in meiosis, including meiotic DNA replication and initiation of recombination (Stuart and Wittenberg 1998; Smith et al. 2001; Benjamin et al. 2003). Recent studies point to Mer2 (also known as Rec107) as a conduit for CDK-dependent regulatory signals controlling DSB formation (Henderson et al. 2006). Mer2 is phosphorylated during meiosis in a CDK/Clb5/Clb6-dependent manner and can be directly phosphorylated by CDK *in vitro*. One of two CDK target sites on Mer2 protein (Ser-30) is essential for recombination: mutation of this residue to alanine abrogates DSB formation, apparently by altering or eliminating interactions of Mer2 with other DSB proteins (Henderson et al. 2006). It has been proposed that regulation of Mer2 activity by CDK is part of the mechanism that coordinates DSB formation with progression through meiosis. Additional processes that regulate DSB formation are discussed in Sect. 4.

The biochemical function of Mer2 is not clear, however. There are no known homologs of Mer2 in organisms aside from other closely related yeast species (Henderson et al. 2006), and no obvious structural motifs aside from a short stretch of heptad repeat sequence (Rockmill et al. 1995). In two-hybrid analyses, Mer2 interacts with itself, Mei4, Xrs2, and Rec114 (Arora et al. 2004). All of these interactions appear to be influenced at least partially by CDK-dependent phosphorylation of Mer2 (Henderson et al. 2006). Mer2 localizes to discrete foci on meiotic chromosomes prior to DSB formation and neither CDK-dependent phosphorylation nor any of the other DSB proteins are required for Mer2 to associate with chromatin (Henderson et al. 2006; Li et al. 2006).

The expression of Mer2 is controlled in an unusual manner. The message is constitutively expressed but has an intron that is inefficiently spliced because of a noncanonical 5' splice site and because the 5' exon is much longer than is typical in yeast (Nandabalan et al. 1993; Nandabalan and Roeder 1995). A meiosis-specific splicing factor, Mer1, binds to a splicing enhancer sequence within the *MER2* intron (Engbrecht et al. 1991; Nandabalan et al. 1993; Nandabalan and Roeder 1995; Spingola and Ares 2000). Mer1 collaborates with other splicing factors including Bud13, Nam8, and U2 snRNP protein Snu17 to increase *MER2* mRNA splicing efficiency (Nakagawa and Ogawa 1997; Spingola and Ares 2000; Spingola et al. 2004; Scherrer and Spingola 2006). The amount of full-length Mer2 protein is thus substantially upregulated in meiosis even though total message levels only increase ~2-fold (Chu and Herskowitz 1998; Henderson et al. 2006; Li et al. 2006)⁴.

⁴ The splicing of mRNA from two other genes, *MER3* and *SPO70/AMA1* is also regulated by Mer1 (Nakagawa and Ogawa 1999; Davis et al. 2000). Mer3 is a DNA helicase required for normal meiotic recombination (Nakagawa and Ogawa 1999; Mazina et al. 2004) and Ama1 is a meiosis-specific

The protein is not completely meiosis-specific, however. The basal splicing efficiency of reporter mRNA constructs is $\sim 15\text{--}20\%$ in the absence of Mer1 (Spingola and Ares 2000; Scherrer and Spingola 2006). As a consequence, full-length Mer2 protein is also present in vegetative cells, where it associates with chromatin in discrete foci (Henderson et al. 2006; Li et al. 2006). Moreover, the unspliced *MER2* message encodes a 131 amino acid polypeptide containing 105 of the 314 residues of the full-length protein, including one of the known CDK target sites. It is not yet known whether significant amounts of truncated protein are made from this message and, if so, whether it has a function – although a cDNA version of *MER2* lacking the intron fully suppresses the DSB defect of a *mer2 Δ* mutant (Engebrecht et al. 1991). The unspliced message is exported from the nucleus and is a target for nonsense-mediated decay (He et al. 2003; Scherrer and Spingola 2006).

Rec114, Mei4. Relatively little is known about the behaviors and functions of the meiosis-specific Rec114 and Mei4 proteins. They can be grouped together with Mer2 based on two-hybrid and coimmunoprecipitation analyses that reveal interactions between them as well as immunocytological studies that show that they colocalize with one another on meiotic chromosomes (Arora et al. 2004; Henderson et al. 2006; Li et al. 2006), (Maleki et al., submitted). Cytological experiments further indicate that these proteins form complexes that are distinct from those containing Spo11 and Rec102 (Li et al. 2006). Rec114, Mei4, and Mer2 still associate with chromatin in the absence of the other proteins, although Mei4 binding to chromosomes is reduced in *mer2 Δ* (Li et al. 2006). Rec114 is required for Spo11 and Mre11 association with hotspot sequences as assessed by ChIP and for Spo11 self-association as measured by coimmunoprecipitation, but not for binding of Spo11 to chromatin in cytological studies of spread chromosomes (Borde et al. 2004; Prieler et al. 2005; Sasanuma et al. 2007). Rec114 overexpression suppresses DSB formation, suggesting that the balance of the amount of Rec114 relative to other factors is critical (Bishop et al. 1999). Rec114 shares limited sequence homology with the Rec7 protein of *S. pombe* (Malone et al. 1997; Molnar et al. 2001) (Sect. 3.2). No homologs of Mei4 are known outside of species closely related to *S. cerevisiae* (Richard et al. 2005). (The *S. pombe* Mei4 protein is not related by sequence or function to *S. cerevisiae* Mei4.)

What do the DSB proteins do? While there has been significant progress in understanding the behaviors of the *S. cerevisiae* DSB proteins and the interactions among them, no clear picture of their precise roles has yet emerged. Functions could include targeting Spo11 binding to specific sites, activating

component of the cell cycle regulator, the anaphase promoting complex (Cooper et al. 2000). It is not clear why these three genes share this unusual means by which their expression is controlled. More recently, it has been demonstrated that RNA from most, if not all, of the meiotic genes that contain introns are inefficiently spliced in vegetative cells, including the transcriptionally repressed DSB genes *REC102*, *MEI4*, and *REC114* (Juneau et al. 2007). Splicing efficiency of these genes is significantly increased in meiotic cells. The mechanism for this regulation is not understood, but is likely to be distinct from Mer1-dependent splicing control.

Spo11 catalytic activity (Sect. 2.3), and/or coordinating DSB formation with chromatin and higher order chromosome structure (see Sect. 4). Interestingly, most of the DSB proteins are capable of targeting Spo11 activity to new locations when they are fused to a sequence-specific DNA binding protein such as Gal4 (A. Nicolas, personal communication). However, it is not yet known whether such targeting is a physiological role for any of the DSB proteins.

It is also not yet known how these proteins work together to promote DSB formation. It is possible that they are stoichiometric subunits of a discrete DSB-forming holoenzyme. However, many of the recent studies seem inconsistent with this hypothesis. For example, as discussed above, it appears that Rec102 and Rec104 interact to form a single functional unit and, although this putative Rec102-Rec104 complex interacts with other DSB proteins, there are numerous differences between Rec102-Rec104 and the other proteins with respect to their genetic dependencies for protein-protein and protein-chromosome interactions. Similarly, differences in localization, interactions, and functional dependencies distinguish many of the DSB proteins from one another (Kee et al. 2004; Prieler et al. 2005; Li et al. 2006). These studies do not rule out the existence of a distinct DSB-forming holoenzyme, but the emerging picture does support the idea that instead there are distinct functional entities that collaborate to make a DSB. An important challenge now is to understand how these subgroups of proteins (Spo11-Ski8, Rec102-Rec104, Mei4-Mer2-Rec114, and Mre11-Rad50-Xrs2) work together and how they are recruited to the sites where DSBs form.

Evolutionary conservation and divergence. Spo11 orthologs are readily identifiable in widely diverged species because of conservation of core structural domains essential for catalytic activity, but the protein sequence overall is not very well conserved (Keeney 2001; Gadelle et al. 2003) (Fig. 5). Many of the other DSB proteins are conserved even less well. Even for yeasts closely related to *S. cerevisiae*, the meiosis-specific DSB proteins have diverged rapidly while (presumably) retaining their functions in DSB formation (Fig. 5) (Richard et al. 2005). For example, Rec114 homologs in *S. cerevisiae* and the very closely related species *Saccharomyces paradoxus* share only 73.4% amino acid identity (Malone et al. 1997 and our unpublished observations). In contrast, nucleotide sequence across all coding regions in these species is > 88% identical and ~ 65% of proteins have $\geq 90\%$ amino acid identity (Cliften et al. 2001). Indeed, it appears that proteins involved in meiotic recombination are generally among the more rapidly diverging of all cellular proteins (e.g., Ramesh et al. 2005; Richard et al. 2005). In part because of this divergence, it has been difficult (or impossible) to identify clear homologs of many of the DSB proteins in organisms other than those closely related to *S. cerevisiae*. However, forward genetic screens for recombination mutants have uncovered a number of additional DSB factors in several other organisms. These are summarized in Sects. 3.2 and 3.3 below. Exam-

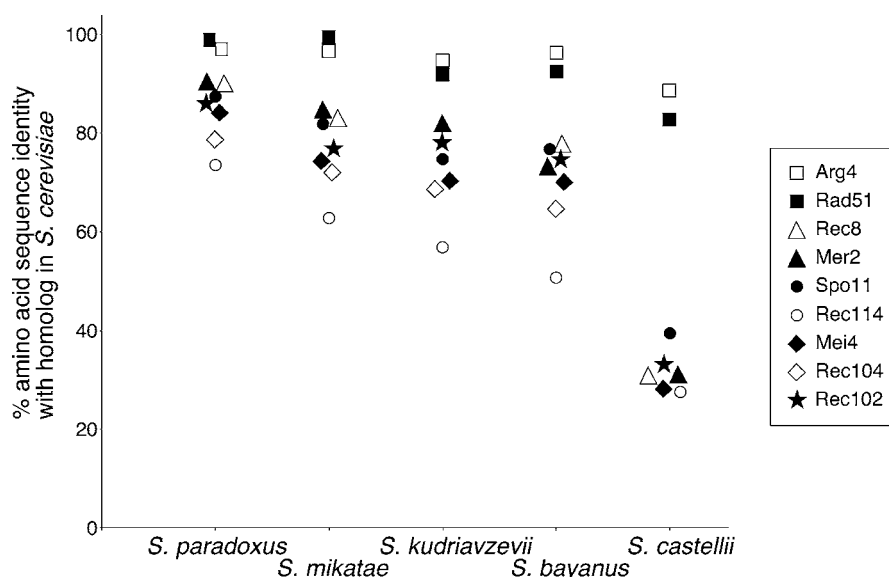


Fig. 5 Rapid divergence of DSB proteins. The sequence conservation (% amino acid identity) between *S. cerevisiae* and each of several other *Saccharomyces* species is shown for the indicated proteins. DSB proteins diverge rapidly, especially when compared with two examples of mitotically expressed proteins, Rad51 and Arg4. Remarkably, Rec104 and Rec114 are less than 80% conserved even in *S. paradoxus*, a very close sibling of *S. cerevisiae*. A Rec104 homolog was not detected in the *S. castellii* genome sequence. Note that this analysis is not meant to imply that DSB proteins are uniquely prone to rapid divergence, although it has been demonstrated that meiotic recombination proteins in general tend to be among the cell's most divergent (Richard et al. 2005). Rather, the point is that they do diverge rapidly and may thus be difficult to identify by routine sequence homology searches in evolutionarily more distant organisms

ples of conserved DSB proteins were discussed along with their *S. cerevisiae* counterparts above (e.g., Spo11 in many organisms; Ski8 in *S. pombe* and *S. macrospora*).

For the proteins that are conserved poorly or not at all, it is possible that there are no equivalents in some organisms or that other proteins are functional substitutes. It is also possible that there has been structural and functional conservation despite insufficient sequence conservation to allow ready identification. However, it is becoming increasingly clear that there are likely to be significant mechanistic differences between organisms, as revealed by the fact that some proteins whose sequences are conserved are not conserved functionally. For example, as noted above, orthologs of Rad50, Mre11, and Xrs2 are not required for DSB formation in *S. pombe*, *C. cinereus*, or *A. thaliana*, although they are required for processing of meiotic DSBs (Gerecke and Zolan 2000; Merino et al. 2000; Bleuyard et al. 2004; Puizina et al. 2004; Young et al. 2004). Similarly, whereas Ski8 orthologs are required

for DSB formation in *S. cerevisiae*, *S. pombe*, and *S. macrospora*, the Ski8 ortholog in *A. thaliana* is dispensable for meiosis (Jolivet et al. 2006). In these examples, it may be that the functions performed in DSB formation in some organisms are performed by different proteins in other species, or that the biochemical functions themselves are only required in certain organisms.

3.2

DSB Proteins in *S. pombe*

Numerous proteins required for DSB formation in *S. pombe* have been identified in addition to Rec12 (the Spo11 ortholog) and Rec14 (Ski8 ortholog). Some of these proteins are conserved, but, as for *S. cerevisiae*, many have no clear homologs in other organisms. Earlier literature on meiotic recombination in *S. pombe* has been reviewed in detail (Fox and Smith 1998; Davis and Smith 2001; Young et al. 2004).

Rec7. The *rec7*⁺ gene was uncovered in a forward genetic screen for meiotic recombination defects (Ponticelli and Smith 1989; De Veaux et al. 1992) and subsequently shown to be required for DSB formation (Cervantes et al. 2000; Davis and Smith 2001). Expression of *rec7*⁺ is induced in meiosis (Lin et al. 1992), and the protein localizes to nuclei and associates with meiotic chromosomes in up to ~ 40 discrete foci that have been proposed to represent sites of recombination initiation (Molnar et al. 2001; Lorenz et al. 2006). The 38.2 kDa protein shares sequence in its N-terminal region with Rec114 (Malone et al. 1997; Molnar et al. 2001), suggesting that these are orthologs.

Mde2, Rec6, Rec15, Rec24, Rec25, Rec27. The *rec6*⁺ and *rec15*⁺ genes were identified in the same screens that yielded mutations in *rec7*⁺, *rec12*⁺, and *rec14*⁺ (Ponticelli and Smith 1989; De Veaux et al. 1992). Expression of both is up-regulated in meiosis (Lin and Smith 1994, 1995), and both are required for DSB formation (Cervantes et al. 2000; Davis and Smith 2001). A function for Rec15 before recombination initiation is suggested by the observation that early meiotic events (including onset of replication) occur earlier than normal in *rec15* mutants (Doll et al. 2005). The molecular nature of this potential early function has not yet been reported, however. Roles for the *mde2*⁺ ("Mei4-dependent"), *rec24*⁺, *rec25*⁺, and *rec27*⁺ genes in DSB formation were uncovered in screens involving systematic deletion of previously uncharacterized genes whose expression is up-regulated in meiosis (Gegan et al. 2005; Martin-Castellanos et al. 2005). All six genes discussed in this section encode small proteins (15.8–40.2 kDa) with no clear homologs in other species and no obvious motifs to suggest biochemical function. Important next steps in understanding the mechanism of DSB formation in *S. pombe* include detailed characterization of the behaviors of these proteins, including testing for chromosomal localization and interactions among them.

Rec8, Rec10, Rec11. Fission yeast does not form synaptonemal complexes, but instead forms filamentous structures called linear elements, which resem-

ble the axial cores of chromosomes in other organisms (reviewed by Loidl 2006). Rec10 is a meiosis-specific protein that localizes to linear elements in immunofluorescence analysis (Lorenz et al. 2004) and is required for linear element formation as assessed by electron microscopy (Molnar et al. 2003). Complete deletion of the *rec10*⁺ coding region confers a phenotype indistinguishable from a *rec12* mutation, thus Rec10 is essential for all DSB formation (Ellermeier and Smith 2005). A *rec10* mutant (*rec10-155*) has been described which does not form linear elements but which does make DSBs, thus indicating that linear elements themselves are not essential for DSBs (Wells et al. 2006). Rec7 foci are closely associated with Rec10-staining linear elements, and these foci do not form in *rec10* mutants (Lorenz et al. 2006). On the basis of these and other observations, it has been suggested that Rec10 recruits Rec7 to chromosomes, although it remains to be determined whether these proteins interact directly, and whether Rec10 is required for recruitment of other DSB proteins to chromosomes as well (Ellermeier and Smith 2005; Lorenz et al. 2006).

Rec8 and Rec11 are meiosis-specific cohesin components that assemble on chromosomes around the time of premeiotic DNA replication (Watanabe and Nurse 1999; Kitajima et al. 2003). DSB formation and meiotic recombination are greatly reduced in the absence of either of these proteins. However, the magnitude of the recombination decrease varies substantially depending on the genomic region assayed, ranging from ~4-fold to >100-fold (DeVeaux and Smith 1994; Krawchuk et al. 1999; Parisi et al. 1999; Ellermeier and Smith 2005). These mutants form aberrant linear elements as assessed by electron microscopy and Rec10 immunostaining (Molnar et al. 1995, 2003; Lorenz et al. 2004), and numbers of Rec7 foci are reduced in *rec8*Δ, consistent with the partial DSB defect (Lorenz et al. 2006). One interpretation of these findings is that the involvement of Rec8 and Rec11 in the loading of Rec7 and perhaps other DSB components is indirect, through formation of Rec10-containing linear elements (Ellermeier and Smith 2005). It has furthermore been suggested that the low level DSB formation in *rec8* and *rec11* mutants is promoted by the presence of residual mitotic cohesin containing Rad21 and Psc3, which are paralogs of Rec8 and Rec11, respectively (Ellermeier and Smith 2005). Interestingly, the regional variation in the *rec8* and *rec11* recombination defects is similar to what is seen with a *rec10* missense mutation (*rec10-109*), leading to the suggestion that this mutant is specifically defective in Rec8-dependent but not Rad21-dependent DSB formation (DeVeaux and Smith 1994; Ellermeier and Smith 2005).

Rec10 is homologous to *S. cerevisiae* Red1 (Lorenz et al. 2004), which similarly localizes to and is required for formation of axial cores (Rockmill and Roeder 1988; Smith and Roeder 1997). Red1 is required for normal DSB formation in *S. cerevisiae*, but this requirement is not absolute as for Rec10 in *S. pombe* (Xu et al. 1997). Rec8 is also conserved in *S. cerevisiae*, but is largely if not completely dispensable for DSB formation in that organism (Klein et al.

1999). Thus, while DSB formation is coordinated with development of higher order chromosome structure in both yeast species (see also Sect. 4.4 below), the molecular details of this coordination appear to differ significantly.

3.3

DSB Proteins in Larger Eukaryotes

The pattern of relatively poor conservation of DSB proteins is even more pronounced moving from fungi to multicellular eukaryotes, where no orthologs are known for most of the budding and fission yeast DSB proteins aside from Spo11. Forward genetic screens in several organisms have uncovered novel proteins required for DSB formation. To date, there are no reported budding or fission yeast orthologs for these proteins and, given the complexity of the DSB forming machinery in yeasts, it is likely that many more genes remain to be uncovered.

***D. melanogaster* MEI-P22.** A *mei-P22* mutation was recovered in a screen for P-element insertions that cause meiotic chromosome missegregation (McKim et al. 1998; Sekelsky et al. 1999). The mutant eliminated gene conversion, crossing over, and recombination nodule formation (McKim et al. 1998), phenotypes that are indistinguishable from those of mutations eliminating the Spo11 ortholog MEI-W68 (McKim et al. 1998; McKim and Hayashi-Hagihara 1998). Direct assays for DSBs are not yet available for *Drosophila*. However, convincing evidence that MEI-P22 is required for DSB formation is provided by observations that strong *mei-P22* mutations: (a) eliminate formation of γ -His2Av, the *Drosophila* equivalent of γ H2AX; (b) suppress the sterility phenotype caused by mutations that affect the repair of DSBs; and (c) can in turn be partially suppressed by X-irradiation (Liu et al. 2002; Jang et al. 2003; Mehrotra and McKim 2006). MEI-P22 is a basic, 35.7-kDa protein with no obvious homologs or structural motifs. The protein localizes to meiotic chromosomes and forms discrete foci prior to appearance of γ -His2Av (Liu et al. 2002; Mehrotra and McKim 2006).

***C. elegans* HIM-17.** Null mutations in *him-17* cause recombination defects similar to *spo-11* mutants in this organism, including defects in chiasma formation, chromosome missegregation, and failure to accumulate RAD-51 foci, and, as for *spo-11* mutants, these defects are suppressed by introduction of radiation-induced DNA damage (Reddy and Villeneuve 2004). HIM-17 protein has six repeats of a sequence motif found in P-element transposase and a number of other proteins (Reddy and Villeneuve 2004); this motif is implicated in DNA binding (e.g., Clouaire et al. 2005), and indeed HIM-17 is chromosome-associated (Reddy and Villeneuve 2004). A role for this protein in post-translational histone modifications is also indicated, because *him-17* null mutants show reduced and delayed accumulation of histone H3 dimethyl lysine 9. This defect is not seen in *spo-11* mutants, so this is not simply a consequence of failing to make DSBs (Reddy and Villeneuve 2004). How HIM-17

functions in DSB formation is not yet clear, however, and it remains to be determined whether its roles in histone modifications and DSB formation are related.

Mouse MEI1. Schimenti and colleagues undertook a forward genetic screen for fertility mutants in mice, using chemical mutagenesis of animals or of embryonic stem cells (Ward et al. 2003). One of the fertility mutants uncovered in this screen is defective for the *Mei1* gene (Libby et al. 2002, 2003). *Mei1* mutations confer phenotypes indistinguishable from *Spo11* mutants in this organism, including chromosome synapsis defects, achiasmate chromosomes in oocytes, absent or reduced formation of RAD51 foci and γ H2AX, and suppression of specific meiotic phenotypes caused by defects in the repair of DSBs (i.e., epistasis to a *Dmc1* mutation) (Libby et al. 2002, 2003; Reinholdt and Schimenti 2005). *Mei1* expression is highly restricted to gonads, and likely orthologs are present in other vertebrates (Libby et al. 2003). Interestingly, *MEI1* sequence polymorphisms in human may be associated with some cases of azoospermia caused by meiotic arrest (Sato et al. 2006). A potential *Mei1* homolog has also been found in *Arabidopsis* (Grelon et al. 2003), but homologs are apparently not present in flies, worms, or fungi (Libby et al. 2003). The biochemical function of the protein in DSB formation remains to be determined.

***A. thaliana* SWI1.** Mutation of the *SWI1* gene (also known as *DYAD*) causes a defect in assembly of RAD51 foci on meiotic chromosomes and rescues the chromosome fragmentation phenotype of a recombination-defective *dif1-1* mutant, strongly indicating that SWI1 is required for initiation of meiotic recombination (Mercier et al. 2003). Two *SWI1* transcripts are produced, capable of encoding proteins of 66 and 73 kDa that differ with respect to presence or absence of N-terminal sequence (Mercier et al. 2001). The proteins contain a potential coiled coil region, but no strong sequence similarity with other proteins has been reported for organisms other than plants (Mercier et al. 2001). The protein is present only very early in meiosis (prior to and during DNA replication) and is chromatin associated (Mercier et al. 2001, 2003). The *swi1* mutants are sterile and also show alterations in meiotic chromosome behavior not seen with *Atspo11* mutations, including defects in axial element formation and sister chromatid cohesion (Mercier et al. 2001, 2003; Agashe et al. 2002). The precise function(s) of this protein and how the different mutant phenotypes are related remain to be determined.

4

Regulation of DSB Formation

Recombination initiation is subject to many layers of control that restrict the timing and location of DSBs and that coordinate DSB formation with other aspects of meiotic chromosome dynamics. Although the mechanisms behind

these controls are poorly understood, recent studies have provided new insights.

4.1

Nonrandom Distribution of DSBs Along Chromosomes

Studies in many organisms demonstrate that meiotic crossovers are more likely to occur in some parts of the genome than in others (reviewed in Petes 2001; de Massy 2003; Kauppi et al. 2004). Because DSBs can be directly mapped in *S. cerevisiae*, it is known that a primary determinant of the crossover distribution in this organism is the distribution of Spo11-dependent DSBs (Wu and Lichten 1994; Baudat and Nicolas 1997; Gerton et al. 2000), although DSB frequencies are much less well correlated with crossover frequencies in *S. pombe* (Young et al. 2002).

DSB distributions in *S. cerevisiae* display multiple levels of spatial organization. There are hot and cold domains that span large portions of chromosomes (on the order of 100 kb) (Zenvirth et al. 1992; Baudat and Nicolas 1997; Gerton et al. 2000). Within these domains, DSBs form in localized hotspots that appear as clusters of frequently cleaved sequences (typically spanning ~70–250 base pairs) surrounded by sequences in which breaks form rarely, if at all (de Massy et al. 1995; Liu et al. 1995; Xu and Kleckner 1995; Xu and Petes 1996; Baudat and Nicolas 1997). Localized hotspots are a feature of meiotic recombination in *S. pombe*, mouse, and human as well, but the factors that determine whether a given DNA sequence will be a DSB hotspot are not well understood in any organism. Detailed reviews have been provided elsewhere (Lichten and Goldman 1995; Fox and Smith 1998; Wahls 1998; Petes 2001; de Massy 2003; Kauppi et al. 2004).

Interestingly, Spo11 can be targeted to novel sites in the *S. cerevisiae* genome simply by fusing the protein to a sequence-specific DNA binding domain, such as that of the Gal4 transcription factor (Peciña et al. 2002). Gal4BD-Spo11 still makes DSBs at most of the normal DSB sites, but more importantly, new DSB sites also appear near Gal4 binding sequences, including sites that lie within larger domains where DSB formation is typically very low (Gerton et al. 2000; Peciña et al. 2002; Robine et al. 2007). DSBs made by Gal4BD-Spo11 still require all of the known DSB proteins (Peciña et al. 2002). However, not all of the binding sites targeted by Gal4BD-Spo11 are capable of giving rise to a DSB, suggesting that targeting of Spo11 to a particular region is not sufficient to recruit all other necessary factors (Robine et al. 2007).

4.2

Cell Cycle Control

DSB formation in *S. cerevisiae* occurs only within a narrow window of time during prophase of the first meiotic division (Padmore et al. 1991). Although

the mechanisms that control this timing are not well understood, several features are clear. One is the meiosis-specific accumulation of Spo11 and other proteins required for DSB formation. Another is the direct regulation of DSB formation by CDK-dependent phosphorylation of Mer2 (Smith et al. 2001; Benjamin et al. 2003; Henderson et al. 2006) (see Sect. 3.1). A third is the coordination of DSB formation with local replication of DNA (see Sect. 4.3 below). Many features remain to be determined, however. First, it is not known whether CDK influences DSB formation in other ways as well (i.e., independent of Mer2). Second, the regulatory kinase Cdc7 is also required for DSB formation and thus for recombination (Schild and Byers 1978; Wan et al. 2006). Cdc7 controls DNA replication origin firing in vegetative cells (reviewed by Sclafani 2000; Masai and Arai 2002) and is required for timely and efficient replication in meiosis (Valentin et al. 2006; Wan et al. 2006). The relevant target(s) of Cdc7 kinase activity that are important for meiotic recombination initiation have not yet been reported. Third, even less is understood about the mechanisms that terminate the time when DSBs can form. All of the DSB proteins persist on chromosomes past the time of DSB formation (Kee et al. 2004; Prieler et al. 2005; Li et al. 2006), but how Spo11 is prevented from acting is not known.

DSB timing in *S. pombe* is similarly restricted to a narrow window during prophase I (Cervantes et al. 2000), and at least some of this restriction is likely due to the meiosis-specific expression of Rec12, Rec6, and other factors (Yamamoto et al. 1997; Davis and Smith 2001). Cell cycle regulatory factors have also been implicated in controlling DSB formation. Specifically, gene conversion is reduced ~2-5-fold in the absence of a meiosis-specific cyclin, Rem1 (although direct measurement of effects on DSB formation have not been reported) (Malapeira et al. 2005). Rem1 is partially redundant with the vegetative B-type cyclin Cig2 for controlling premeiotic DNA replication (Borgne et al. 2002; Malapeira et al. 2005), so it is possible that these cyclins are also redundant for controlling recombination, perhaps analogous to redundancy of Clb5 and Clb6 cyclins in *S. cerevisiae* (Stuart and Wittenberg 1998; Smith et al. 2001). Moreover, the Hsk1 kinase (the ortholog of Cdc7) is essential for DSB formation, so this means of regulating recombination initiation also appears to be conserved (Ogino et al. 2006). Direct targets of Hsk1 (and possibly of CDK) in DSB formation remain to be determined.

On the basis of cytological analyses, DSB formation is restricted to early meiotic prophase in other organisms as well. For example, Spo11-dependent γ H2AX appears on mouse spermatocyte chromosomes during the leptotene stage (Mahadevaiah et al. 2001). Detailed timing patterns are not universal, however, as γ -His2Av formation in *Drosophila* oocytes does not occur until later in prophase, when SC formation is nearly complete (Jang et al. 2003; Mehrotra and McKim 2006). Regardless of whether DSBs occur relatively early or relatively late, what controls DSB timing in these and other organisms is not yet understood, and in particular whether DSBs are controlled by cell

cycle regulatory factors is not known. In this context, however, it is interesting to note that *Drosophila* MEI-P22 has nine matches to the CDK consensus phosphorylation site, so it is tempting to speculate that CDK may directly control MEI-P22 activity.

4.3

DNA Replication

In order for meiotic recombination to physically connect homologous chromosomes, recombination must occur between homologous nonsister chromatids, and sister chromatid cohesion along chromosome arms must be maintained until the first meiotic division (Petronczki et al. 2003). This feature of meiotic chromosome biology places an important temporal constraint on DSB formation, namely, that DSBs must occur only in chromosomal segments that have already been replicated. Until recently, it was thought that there is a strict functional dependency in *S. cerevisiae* whereby DSB formation can only occur after replication fork passage (Borde et al. 2000; Baudat and Keeney 2001; Smith et al. 2001). This view was based in part on observations that recombination initiation is blocked when DNA replication is prevented by mutations (e.g., *clb5/clb6* Δ , *cdc21*, *pol1*) or chemical inhibitors (e.g., hydroxyurea) (Schild and Byers 1978; Budd et al. 1989; Borde et al. 2000). There are several complications with these studies however. For example, hydroxyurea treatment blocks induction of early meiotic genes, including *SPO11* itself (Lamb and Mitchell 2001). Also, CDK is now known to directly regulate DSB formation through phosphorylation of Mer2 (Henderson et al. 2006) in addition to controlling meiotic DNA replication (Stuart and Wittenberg 1998). And checkpoint responses that arrest cell cycle progression are induced by certain mutations that cause replication defects after replication origins have fired (e.g., mutations affecting DNA polymerase α (*pol1*) or thymidylate synthase (*cdc21*)). In principle, activating these checkpoints could cause a regulatory block to DSB formation (Hochwagen and Amon 2006), and indeed this appears to be the case in *S. pombe* because eliminating replication checkpoint genes in this organism permits recombination initiation even when DNA synthesis is inhibited (Tonami et al. 2005; Ogino and Masai 2006). Because of these considerations, the failure of *S. cerevisiae* cells to make DSBs in these cases cannot be ascribed solely to absence of DNA replication. Finally, when the *S. cerevisiae* replication factor Cdc6 is not expressed in meiotic cells, DSBs are formed even though DNA replication appears not to be initiated (Hochwagen et al. 2005), similar to the previously demonstrated ability of *S. pombe* cells to initiate meiotic recombination when replication factors are depleted (Murakami and Nurse 2001). Thus, in both *S. pombe* and *S. cerevisiae*, DNA replication is not a strict prerequisite for DSB formation.

Nevertheless, there is strong evidence that DNA replication and DSB formation are closely connected in normal *S. cerevisiae* cells, from experiments

in which replication on the left arm of chromosome III was delayed by deletion of replication origins on that arm or by truncating the arm to bring the telomere close to the sequences normally in the middle of the arm (Borde et al. 2000). In these strains, the delay in replication timing was matched by a corresponding delay in the time of DSB formation by Spo11, such that DSBs always formed ~ 90 min after passage of the replication fork. It is possible that replication fork passage promotes establishment of chromosomal features (chromatin or higher order chromosome structures) that constrain subsequent DSB formation (Cha et al. 2000). An alternative possibility is that replication origins and DSB sites compete for a rate-limiting factor(s). If origins compete more effectively and sequester these factors, then DSB sites would not be acted upon until the factors were released locally upon origin firing, or perhaps as the factors track with the replication fork. If pre-replicative complexes never form at origins (e.g., in the absence of Cdc6), then there would be no competition and the rate-limiting factors would be available to activate DSB formation. Cdc28-Clb5/Clb6 and Cdc7-Dbf4 are attractive candidates for such rate-limiting factors (Henderson et al. 2006; Ogino et al. 2006; Wan et al. 2006).

4.4

Higher Order Chromosome Structure

Meiotic chromosomes are organized into linear arrays of chromatin loops, with the loop bases and associated proteins defining a structural axis for each chromatid (reviewed in Kleckner 2006). It appears that meiotic DSBs occur preferentially in the sequences on the loops (Blat et al. 2002), but the recombination complexes that carry out strand exchange and other reactions are associated with the axes (Moens et al. 1998). It has been proposed that protein-DNA complexes at DSB sites are recruited to chromosome axes, either as a condition for DNA cleavage or as a consequence of DSB formation (van Heemst and Heyting 2000; Blat et al. 2002). Supporting this view, DSB formation is partially defective in *S. cerevisiae* mutants lacking certain chromosome structural components (Red1 and Hop1) (reviewed in Keeney 2001; Hunter 2007). As discussed in Sect. 3.2, an even more severe DSB defect is seen in *S. pombe* mutants lacking Rec10 (Lorenz et al. 2004; Wells et al. 2006). The molecular events that connect DSB formation with chromosome axes are not well understood. However, in *S. cerevisiae*, overexpression of Rec104 can suppress DSB defects caused by *hop1* mutation, suggesting that the Rec102/Rec104 complex may be involved in this process (Friedman et al. 1994). And in *S. pombe*, colocalization of Rec7 with linear elements may suggest involvement of this protein (Lorenz et al. 2006).

In *Drosophila* oocytes, DSB formation appears closely coordinated with formation of the synaptonemal complex: γ -His2Av appears only after the synaptonemal complex is nearly fully formed, and numbers of γ -His2Av foci

are reduced (but not absent) in mutants defective for C(3)G and C(2)M proteins, which are components of the synaptonemal complex (Jang et al. 2003; Manheim and McKim 2003; Mehrotra and McKim 2006) (but see also Webber et al. 2004). The molecular nature of this dependence remains to be determined. Synaptonemal complex components are not required for DSB formation in other organisms studied, including *S. cerevisiae*, *C. elegans*, mouse, and plants (e.g., Sym et al. 1993; Colaiacovo et al. 2003; de Vries et al. 2005; Higgins et al. 2005).

5

A Model for the Mechanism of DSB Formation in *S. cerevisiae*

A working model for steps immediately prior to and just after DSB formation is shown in Fig. 6. This model is based on a previous proposal (Keeney 2001), with modifications suggested by more recent studies (especially Neale et al. 2005; Prieler et al. 2005). The first step is the assembly of a Spo11-containing complex on DNA (Fig. 6a). This complex likely contains Spo11, Ski8, Rec102, Rec104, and Mre11 and may contain some or all of the other DSB proteins. These complexes are not sufficient to generate a DSB, but must instead be activated (Prieler et al. 2005). In wild-type cells, activation of Spo11-DNA complexes drives them to carry out concerted reactions in which Spo11 cleaves the DNA and Mre11 introduces single-strand nicks (Fig. 6b,c), followed rapidly by resection (Fig. 6d). This series of steps is envisioned to be the irreversible process that commits this DNA segment to undergoing homologous recombination. It is attractive to propose that DSB formation and processing are concerted reactions because the coupling of these events may provide a means to ensure that DSBs are efficiently repaired.

The biochemical nature of the putative activation step is not clear. One possibility is that there are multiple sub-steps. For example, Spo11 might undergo a conformational change that moves the catalytic tyrosine residues into position to interact with the metal binding pockets (Fig. 6b), analogous to conformational changes proposed for TopoVI (see Fig. 3) (Nichols et al. 1999; Corbett and Berger 2004). Such a change might poise Spo11 to cleave the DNA in response to other signals (see below), and might be effected by binding of additional DSB proteins, post-translational modifications by CDK or other regulatory molecules, and/or ATP-dependent movements by Rad50 or other proteins. Each step up until DNA strand cleavage could in principle be reversible such that only a subset of activated complexes actually gives rise to a DSB. Indeed, because Spo11 is in excess over DSB formation, it is reasonable to think that only a few Spo11-DNA complexes are selected to proceed at each step.

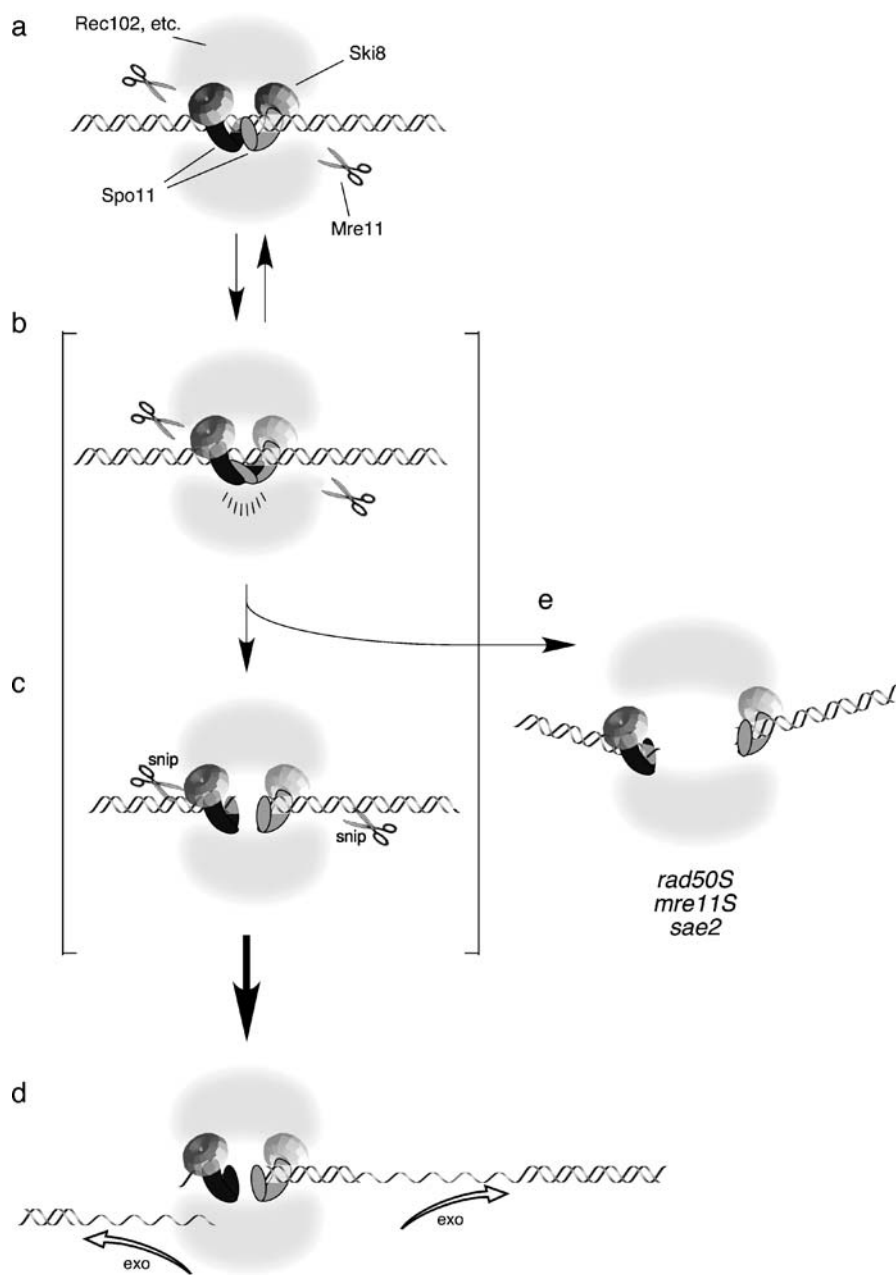
If this scheme is correct, what is the ultimate trigger for DSB formation? One hypothesis is that mechanical stress along the chromosome drives this

reaction. Kleckner and colleagues have proposed that mechanical stress is generated by expansion of chromatin against constraining elements (Borner et al. 2004; Kleckner et al. 2004; Kleckner 2006). This stress then promotes structural and enzymatic processes on meiotic (and mitotic) chromosomes, including crossover formation. This model could be applied to DSB formation if mechanical stress provides a physical force to favor Spo11 dimer disruption and subsequent activation of Mre11 endonuclease (Keeney 2001) (Fig. 6c).

What happens when Mre11 nuclease activity is not available, as in *mre11S* and (presumably) *rad50S* and *sae2* mutants? Perhaps Spo11 complexes undergo the activation step to establish the potential to make a DSB, stabilizing the complex and giving rise to the “tight binding” observed in ChIP experiments (Prieler et al. 2005). The “tight binding” might reflect stabilization of Spo11 on the DNA by other DSB proteins forming a clamp-like higher order structure (Fig. 6). However, in the absence of Mre11 nuclease, the complexes cannot carry out concerted Spo11-mediated and Mre11-mediated DNA strand cleavages, so they remain arrested in a quasi-stable state that eventually decays to give an irreversible DSB, perhaps from physical disruption of the Spo11 dimer (Fig. 6e). In this scenario, low stress levels are sufficient to drive DSB formation in wild-type cells, but higher stress levels are required to generate irreversible DSBs in *rad50S* mutants. In this scheme, *rad50S* DSBs are not on the normal DSB pathway, as has been previously suggested (Prieler et al. 2005).

Application of the stress model to DSB formation is attractive because it provides a way to account for the effects of chromosome structure mutants on DSB formation (Sect. 4.4). This model also explains why DSBs appear to interfere with one another (Wu and Lichten 1995; M. Lichten, personal communication). This is because the use of stress to drive a reaction results in local relief of the stress. Thus, separate occurrences of the same reaction nearby are inhibited (Kleckner et al. 2004).

This hypothesis is also attractive because it provides an explanation for the evolutionary curiosity of why Spo11 is Spo11, that is to say, why meiotic recombination is initiated by a topoisomerase relative instead of a nuclease. A number of studies have shown that meiotic recombination can be supported by DNA damage mechanisms that do not involve Spo11. For example, DSBs made by site-specific endonucleases induce recombination that has many of the hallmarks of normal meiotic recombination (Malkova et al. 2000; Neale et al. 2002). Moreover, DSBs caused by ionizing radiation can at least partially suppress meiotic inviability of *spo11* mutants in many organisms (Thorne and Byers 1993; Dernburg et al. 1998; Celerin et al. 2000; Liu et al. 2002; Storlazzi et al. 2003). Perhaps most strikingly, DNA strand discontinuities caused by elimination of the flap endonuclease Rad2 in *S. pombe* are sufficient to restore meiotic recombination and spore viability of a *rec12* mutant to near normal levels (Farah et al. 2005a). Why then is Spo11 a universal feature of meiotic recombination even though other mechanisms can



- ◀ **Fig. 6** Model for the mechanism of DSB formation. Spo11 is represented as for TopoVIA in Fig. 3. The WD repeat protein Ski8 is shown as a donut bound to Spo11, Mre11 endonuclease as a pair of scissors, and other DSB proteins (“Rec102, etc.”) as clouds encompassing the Spo11 complex on DNA. Key features of this model are that Spo11 binding to DNA and activation to cleave are separate steps (Prieler et al. 2005); DNA cleavage by Spo11 and the onset of DSB processing by Mre11 (with or without other nucleases) are concerted reactions; and the irreversible Spo11-bound DSBs in *rad50S* and other mutants are off-pathway (Prieler et al. 2005). **a** Assembly of a pre-DSB complex on DNA, consisting of Spo11, Ski8, and at least a subset of the other DSB proteins. **b,c** Activation of Spo11 to cleave DNA. In panel b, conformational change within the complex (perhaps within Spo11 itself) renders Spo11 competent to cleave DNA, but it has not yet done so. This complex may be reversed without forming a DSB. In panel c, activated Spo11 complexes are competent to be driven forward: Spo11 forms a DSB and, in a concerted reaction, Mre11 nicks the DNA to initiate processing of the DSB. This sub-step is irreversible and commits this site to undergoing homologous recombination. **d** Exonucleolytic processing. Further degradation of the 5' terminal strands follows rapidly and in a concerted manner upon DSB formation and Mre11 nucleolytic activity. **e** Irreversible DSB formation in *rad50S*, *mre11S*, and *sae2* mutants. Activation of Spo11 in the absence of Mre11 nuclease activity stabilizes the complex (Prieler et al. 2005), but a frank DSB cannot form because concerted processing by Mre11 cannot occur. This complex eventually decays to give an irreversible DSB that is related to normal meiotic DSBs but that is off the normal pathway

substitute? This universality suggests that a topoisomerase-like mechanism is important, not just DNA cleavage per se. I propose that this is because topoisomerases (unlike nucleases, which act hydrolytically) have inherently stress-sensitive catalytic activities: application of mechanical force can in principle drive the cleavage reaction toward irreversibility. Indeed, in single molecule experiments of the decatenation activity of *E. coli* TopoIV or *Drosophila* TopoII, application of force to the DNA substrate caused the enzymes to stall, attributed to inhibition of the ability to reseal DNA ends (Strick et al. 2000; Stone et al. 2003).

Although there has been significant progress in understanding the mechanism and control of Spo11 activity, much remains to be learned. One challenge that has not yet been met is the development of biochemically tractable systems for studying meiotic recombination initiation. Another challenge is the need to fully catalog Spo11-associated proteins in organisms other than yeasts. The existing studies make it clear, however, that the complexities of meiotic recombination initiation will continue to keep students of this fascinating process busy for many years to come.

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Synapsis, Double-Strand Breaks, and Domains of Crossover Control in *Drosophila* Females

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Abstract *Drosophila* is an attractive model system in which powerful tools in genetics and cytology can be used to identify and characterize the genes required for meiotic recombination. This article reviews recent developments in understanding how pairing and synapsis proceed in the absence of double-strand breaks (DSBs), the relationship of DSB formation to synapsis, how crossovers are determined and formed, and the role that chromosome structure, including specialized sites, plays in regulating DSB formation and repair.

Abbreviations

DSB double-strand break
EM electron microscopy
FISH fluorescence in situ hybridization
mei- meiotic
mus- mutagen sensitive
PMS postmeiotic segregation
RN recombination nodule
SC synaptonemal complex

1

Introduction

Drosophila holds a special place in the study of meiosis, since many of the basic principles of genetics, including the first linkage maps, were established using this model organism (Sturtevant 1913). The importance of crossing over for segregation was recognized through the analysis of meiotic mutants and nondisjunction events in *Drosophila* (Baker et al. 1976a,b; Bridges 1916; Cooper 1948). More recently, the meiotic recombination pathway including the pairing and disjunction of the homologous chromosomes has been elaborated in much detail.

Many aspects of the pairing and recombination processes in *Drosophila* are similar to those of other organisms. The recent advances in these areas,

Table 1 Summary of genes known to be involved in meiotic recombination in *Drosophila*

Genes	Class	Orthologs ^a	DSB formation in null mutant	DSB repair in null mutant	Crossing over in null mutant	Refs.
<i>c(3)G</i>	SC	Zip1/Syp1	Reduced	Normal	None	Hall 1972; Page and Hawley 2001
<i>c(2)M</i>	SC	Kleisin	Reduced	Normal	Reduced	Manheim and McKim 2003
<i>mei-P22</i>	DSB formation	None	None	Normal	None	Liu et al. 2002
<i>mei-W68</i>	DSB formation	Spo11	None	Normal	None	McKim and Hayashi-Hagihara 1998
<i>ord</i>	Sister chromatid cohesion	None	Normal	Normal	Reduced	Bickel et al. 1996, 2002
<i>pds5</i>	Sister chromatid cohesion	Spo76/Pds5	Normal	Delayed	Lethal	Mehrotra and McKim 2006
	DSB repair					
<i>spn-A</i>	DSB repair	Rad51	Normal	Delayed	Sterile	Staeva-Vieira et al. 2003
<i>spn-B</i>	DSB repair	Rad51-like/XRCC3	Normal	Delayed	Reduced	Ghabrial et al. 1998
<i>spn-C</i>	DSB repair	HEL308		Delayed	Sterile	McCaffrey et al. 2006
<i>spn-D</i>	DSB repair	Rad51-like/Rad51C	Normal	Delayed	Reduced	Abdu et al. 2003; Ghabrial et al. 1998
<i>okr</i>	DSB repair	Rad54	Normal	Delayed	Reduced	Bhagat et al. 2004; Ghabrial et al. 1998
<i>rad50</i>	DSB repair	Rad50	Normal	Delayed	Lethal	Gorski et al. 2004; Mehrotra and McKim 2006
<i>mre11</i>	DSB repair	Mre11	Normal	Delayed	Lethal	Giapponi et al. 2004
<i>mei-218</i>	Precondition	Mcm-like	Normal	Normal	Reduced	McKim et al. 1996
<i>mei-217</i>	Precondition		Normal	Normal	Reduced	Liu et al. 2000
<i>rec</i>	Precondition	Mcm-8	Normal	Normal	Reduced	Blanton et al. 2005
<i>mcm5^b</i>	Precondition	Mcm-5	Normal	Normal	Reduced	R. S. Hawley, per. commun.

Table 1 (continued)

Genes	Class	Checkpoint response	Orthologs ^a	DSB formation in null mutant	DSB repair in null mutant	Crossing over in null mutant	Refs.
<i>mei-41</i>	Checkpoint response		ATR/MEC1	Normal	Delayed	Reduced	Ghabrial and Schupbach 1999; Hari et al. 1995
<i>mei-9</i>	Exchange class		Rad1	Normal	Normal	Reduced	Sekelsky et al. 1995; Yildiz et al. 2002
<i>mus312</i>	Exchange class			Normal	Normal	Reduced	Yildiz et al. 2002
<i>ERCC1</i>	Exchange class		ERCC1/Rad10	Normal	Normal	Reduced	Radford et al. 2005

^a Representative orthologs from mammals or *S. cerevisiae* are given
^b Data are for a special allele, null alleles are lethal

including the genes which have been characterized (Table 1), are summarized in this chapter. Important differences that exist between the meiotic recombination pathways in different organisms are also emphasized. Most notable are the differences in the relationship between synapsis and recombination of homologous chromosomes. Synapsis, as marked by the close and precise alignment of the homologs and mediated by the synaptonemal complex (SC), is one of the most recognizable features of meiosis (Fig. 1). Most organisms exhibit an important relationship between SC formation and recombination. Double-strand breaks (DSBs), the initiators of meiotic recombination, are major determinants of stable juxtaposition of homologs and a prerequisite for SC formation (Henderson and Keeney 2004; Keeney 2001;

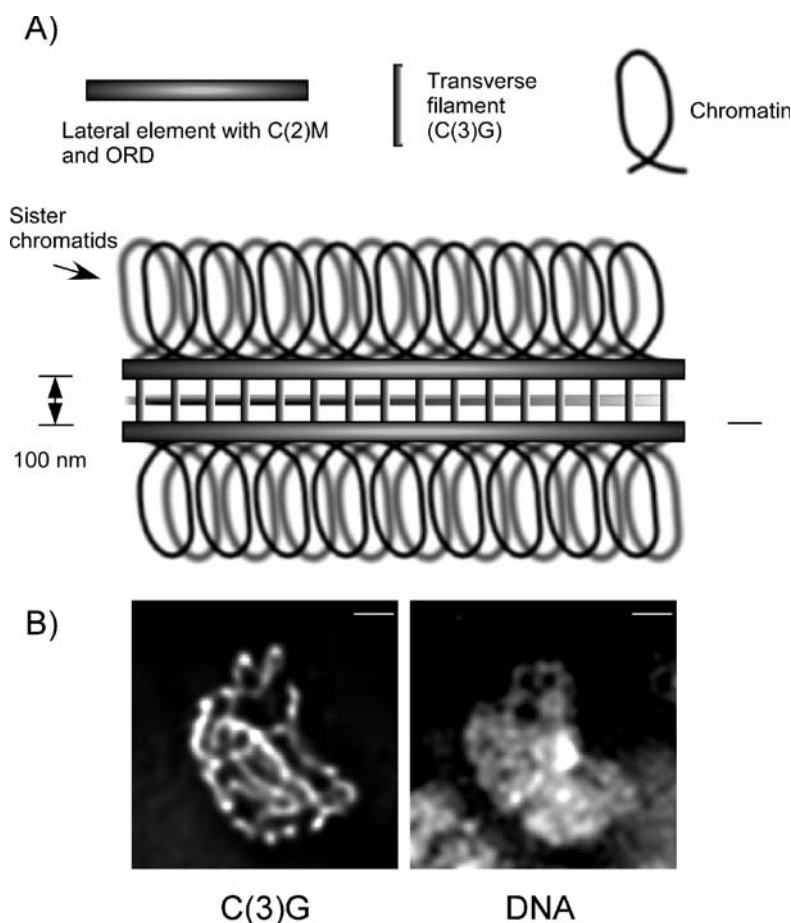


Fig. 1 Structure of the synaptonemal complex. **a** Schematic of synapsed chromosomes at pachytene. Cohesion between sister chromatids is not shown. **b** A single oocyte stained for C(3)G and DNA. The scale bar represents 1 μ m

see S. Keeney, this BOOK). Yet, in a few organisms, such as *D. melanogaster* and *Caenorhabditis elegans*, SC forms in the absence of DSBs (Dernburg et al. 1998; McKim et al. 1998). Thus, *Drosophila* provides an interesting alternative for studying the mechanisms that bring and hold the homologs together.

The SC has an important relationship with crossing over and is required for crossovers in most organisms, including *Drosophila*. How the differences in the relationship between DSBs and synapsis noted above affect the function of SC proteins in crossing over is not known. *Drosophila*, however, does seem to have developed a novel mechanism for producing crossovers while dispensing some of the proteins commonly used by other organisms.

2

The System: An Orderly Series of Meiotic Events

Meiosis is one of the first programmed events in the process of oocyte differentiation and occurs in the context of oocyte development. *Drosophila* females have a pair of ovaries and each is comprised of several tubes (or ovarioles) of developing oocytes. At the anterior end of each ovariole is the germarium (Fig. 2) where the stem cells give rise to specialized cells called cystoblasts. The cystoblasts undergo four successive incomplete mitotic divisions producing a 16-cell cyst with intercellular junctions called ring canals. Premeiotic DNA replication then follows, which appears to be longer than the preceding mitotic S-phases (Carpenter 1981), and then meiotic prophase and recombination are initiated. As the 16-cell cyst moves toward the posterior end of the germarium, two pro-oocytes enter pachytene. Eventually, only one of these two pro-oocytes is selected to become the oocyte and the remaining 15 cells develop with a nurse cell fate.

The germarium is divided into four regions based on the morphology of the 16-cell cysts (Fig. 2). The first 16-cell cysts appear following the last premeiotic division in region 2a of the germarium. Throughout regions 2a and 2b, most of the oocytes are in pachytene with SC assembled between homologs along their entire lengths. Also at this time, two kinds of recombination nodule (RN) have been observed. First are the ellipsoidal or early RNs, which are randomly distributed along the bivalents and depend on the *Drosophila* Spo11 ortholog MEI-W68 (Carpenter 2003; McKim et al. 1998). Second are the spherical or late RNs, which begin to appear later but overlap in time with the early RNs. The late RNs may mark the sites of crossing over (see below).

By region 3, oocyte determination has been completed since a single oocyte is usually evident and positioned toward the posterior of the cyst. Even though the cysts in regions 2a–3 are arrayed in the order of their developmental ages, their absolute position in the germarium does not necessarily

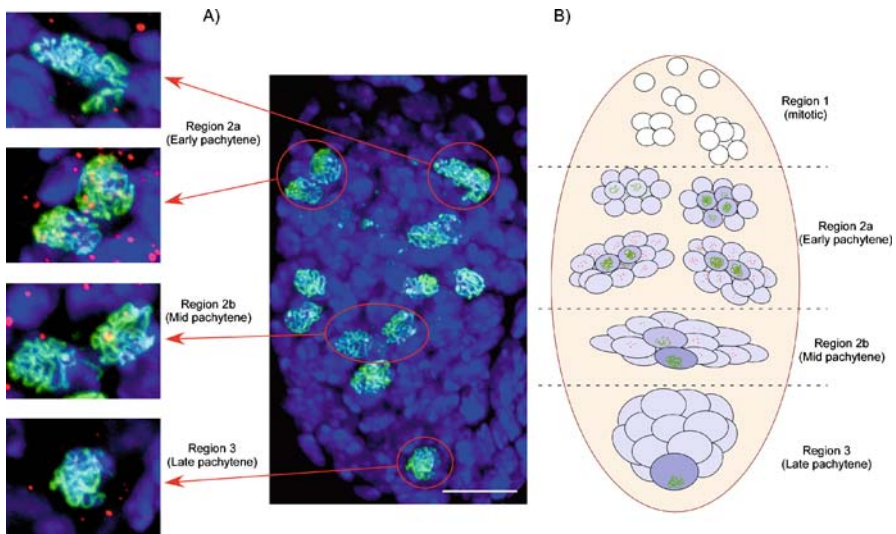


Fig. 2 The *Drosophila* germarium. **A** Confocal image of a *Drosophila* germarium with DNA in blue and SC (C(3)G) in green. DSBs (γ -His2Av) in red are only shown in the magnified images. The anterior end is toward the top and includes region 1, where the cystoblasts undergo four incomplete mitotic divisions to form the 16-cell cyst. In region 2a, the two pro-oocytes within each 16-cell cyst move rapidly through zygotene and most are in pachytene. The remaining 14 nurse cells stain weakly for SC markers but do experience DSBs. In the earliest pachytene pro-oocytes, there are few if any γ -His2Av foci. By region 3, the single oocyte has been determined. "Late" pachytene persists into the vitellarium and is defined by the absence of γ -His2Av. **B** Schematic of *Drosophila* germarium. In addition to SC in green and γ -His2Av foci in red, ORB, a cytoplasmic oocyte marker (Lantz et al. 1994) is shown in blue

equate to their specific meiotic stages. Within a germarium, however, several successive stages of meiotic recombination and oocyte development can be observed in temporal order. Combining this attractive cytology and the availability of meiotic mutants, several genes required for pairing and synapsis of homologous chromosomes and the formation of crossovers in the *Drosophila* female germline have been characterized.

3 Homolog Recognition or Alignment

3.1 Premeiotic and Somatic Pairing

One reason why DSBs are not required for homolog pairing in *Drosophila* could be that the homologous chromosomes are already aligned before the

initiation of meiosis. Premeiotic pairing can be defined as the associations of homologs that precede the onset of meiotic S-phase (Grell and Day 1970). In *Drosophila*, like most Dipterans, homologous pairing is observed throughout most of the life cycle of the organism and is known as somatic pairing (Fung et al. 1998; Hiraoka et al. 1993). It involves multiple interstitial interactions along chromosomes; however, their molecular nature remains to be resolved (Fung et al. 1998; Marshall et al. 1996). The ability to pair and synapse homologs by DSB-independent means in *Drosophila* could be due to mechanisms similar to those involved in somatic pairing.

Premeiotic contacts are lost during DNA replication and they reappear at the onset of leptotene (Csink and Henikoff 1998; Fung et al. 1998; Weiner and Kleckner 1994). Thus, there are uncertainties as to what features are shared between somatic pairing, germline premeiotic pairing, and pairing following premeiotic S-phase in *Drosophila*. Somatic pairing may be disassembled and then reestablished during the initial stages of meiosis or may continue in the female germline into early prophase. These initial contacts could then mature into synapsis. Any somatic pairing mechanism, however, could only bring the meiotic chromosomes closer together. What triggers SC formation remains elusive and is probably the more important question.

Fluorescence in situ hybridization (FISH) to prophase chromosomes has demonstrated that, prior to SC formation, most homologous chromosomes are aligned at a distance of $\leq 0.4 \mu\text{m}$, even in the mitotically dividing premeiotic germline cells (Sherizen et al. 2005). This type of alignment could substitute for presynaptic alignment in other organisms. Interestingly, this pairing is not maintained in *c(3)G* mutants, which lack SC (Gong et al. 2005; Sherizen et al. 2005). Since the homologs are paired prior to SC formation in the wild type, it is likely that homolog pairing becomes dissociated in *c(3)G* mutants. Similarly, in *C. elegans syp-1* mutants (an ortholog of *c(3)G*), homologs that are initially paired dissociate prematurely (Colaiacono et al. 2003; MacQueen et al. 2002). These results suggest that the mechanisms that are involved in premeiotic pairing are not sufficient to maintain meiotic chromosome pairing. SC formation is required to maintain homolog associations during prophase I.

3.2

DSB Independent Mechanisms of SC Formation

Several components of the SC have been identified in *Drosophila* (Fig. 1). C(3)G has sequence characteristics similar to transverse filament proteins in several other organisms (Page and Hawley 2001). C(2)M is related to the Kleisin family of cohesion/condensin proteins which includes Rec8, a meiosis-specific cohesion protein that also has a role in the formation of the lateral element of the SC (Schleiffer et al. 2003). C(2)M colocalizes with C(3)G and is required for its assembly into long threads, consistent with the pro-

posal that C(2)M is part of the SC (Manheim and McKim 2003). C(2)M also interacts with SMC3, strengthening the link to Rec8 and the lateral elements (Heidmann et al. 2004). Immuno-electron microscopy of C(3)G and C(2)M has confirmed most of these structural conclusions (Anderson et al. 2005). C(2)M, however, is not required for sister-chromatid cohesion, suggesting it only carries out the functions in the Kleisin family in SC assembly and crossover formation (Manheim and McKim 2003). Another protein which interacts with the SC is ORD, which unlike C(2)M is required for sister-chromatid cohesion. ORD is also required for normal levels of crossing over and the SC forms abnormally in *ord* mutants (Balicky et al. 2002; Webber et al. 2004). ORD does not have known orthologs in genomes beyond the *Diptera* but does colocalize with C(2)M and C(3)G, and may be closely associated with the lateral elements.

Zygotene is rapid in *Drosophila* and it is not possible to observe the leptotene stage, since the axial elements are not observed prior to synapsis. Indeed, lateral and transverse elements appear at the same locations during zygotene (Carpenter 1975, 1979a). Similarly, C(2)M staining is not observed prior to C(3)G. C(2)M and C(3)G staining appear simultaneously in short stretches at zygotene. Nonetheless, the relationship between these two proteins is like lateral and transverse elements in other organisms. In the absence of C(3)G, C(2)M still assembles into chromosome-associated threads. In contrast, the absence of C(2)M causes C(3)G to accumulate in chromosome-associated patches, as if SC can initiate but not be propagated (Manheim and McKim 2003).

Drosophila mutants that lack meiotic DSBs, such as *mei-W68* or *mei-P22* (see Sect. 4.1), form SC in early pachytene very similar to the wild type (Liu et al. 2002; McKim et al. 1998). No delays or defects in SC formation are observed in these mutants. Since SC is observed before evidence of DSB formation or recombination (Carpenter 1979b; Jang et al. 2003; Mehrotra and McKim 2006), it is conceivable that the SC could have a role in DSB formation. Alternatively, both events could be independent of each other. This is described in more detail below (Sect. 4.2).

3.3

Specialized Sites and Maintenance of Paired Homologs

One of the most intriguing features of meiotic recombination in *Drosophila* is that crossing over is dependent on the structure of the chromosomes. This is most clearly shown in translocation heterozygotes, which are region-specific crossover suppressors in *Drosophila* (Hawley 1980). In translocation heterozygotes, the suppression of crossing over extends for a long physical distance from the breakpoint but is also confined to the interval between two discrete boundaries (Fig. 3). This has led to the hypothesis that *Drosophila* has specialized sites that are involved in recombination, origi-

A) Chromosome 3R: early pachytene



B) Chromosome 3R: middle pachytene



C) Chromosome 3R in a translocation heterozygote

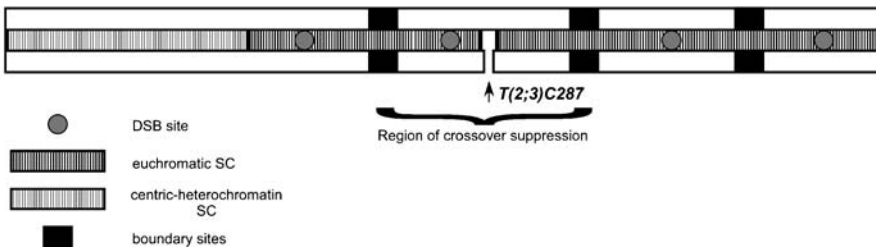


Fig. 3 Schematic of chromosome 3R in pachytene. **A** Chromosomes form SC prior to evidence of DSB formation. **B** An average of about four DSBs form per chromosome arm which may be randomly distributed. Maturation into crossovers depends on several factors, including proximity to the centric heterochromatin (Beadle 1932), presence of SC, and continuity between two boundary sites. Shown here are the three known sites on chromosome 3R (Sherizen et al. 2005). Four sites are known on the X chromosome. **C** A breakpoint, such as *T(2;3)C287*, will inhibit DSBs between the flanking boundary sites from becoming crossovers. The breakpoint has no effect on pairing and only minor effects on SC formation. The breakpoint does break the continuity of the chromosome axis between two boundary sites, which may disrupt a feature of chromosome structure required for crossover formation. This structure could be established by a component of the SC closing in from two sides, although it is not known if boundary sites initiate SC formation. The chromosome 2 portion of *T(2;3)C287* is not shown

nally referred to as “pairing sites,” based on the hypothesis that crossover suppression in translocation heterozygotes was due to defects in homolog pairing (Hawley 1980). The mapping of these sites has been achieved by analyzing the patterns of crossover suppression in a series of translocations with breakpoints spanning the X (Hawley 1980) or third (Sherizen et al. 2005) chromosomes.

Surprisingly, “pairing” might not be the right adjective since no pairing defects associated with crossover suppression have been observed in translocation or inversion heterozygotes (Gong et al. 2005; Sherizen et al. 2005). Therefore, these sites are more appropriately referred to as boundary sites, for the observation that they define the boundaries of crossover suppression in a translocation heterozygote (Fig. 3). These data, namely the accurate pairing

of homologous chromosomes in translocation and inversion heterozygotes, argue against the occurrence of nonhomologous synapsis. This has been observed in rearrangement heterozygotes of some organisms (reviewed by Zickler and Kleckner 1999) including *C. elegans* translocation heterozygotes (Macqueen et al. 2005).

The function of the boundary sites, if any, in synapsis is not known. The FISH studies did not rule out a role for these sites in synapsis. By analogy to the pairing centers in *C. elegans*, these specialized sites could promote interactions between homologs independent of DSB formation, including the initiation of synapsis (Sherizen et al. 2005). In *C. elegans* these sites are involved in stabilization of pairing and the initiation of SC formation (Macqueen et al. 2005). It is important to point out, however, that boundary sites need not be SC initiation sites to explain their effects on crossing over (see Sect. 6.2).

4

Recombination Initiation

4.1

DSB Formation in the Context of SC

Regulation of DSB formation is an essential aspect of meiosis. Once a sufficient number of DSBs have been generated, the process has to be attenuated to prevent excessive damage to the cells. In order to have an assay for DSB during meiosis, an antibody has been used which detects a variant form of a histone 2A His2Av in *Drosophila* (Jang et al. 2003; Mehrotra and McKim 2006). This histone, like H2AX in humans, is phosphorylated at DSB sites (Madigan et al. 2002). In wild-type germaria, γ -His2Av foci induced by meiotic DSB formation appear in early pachytene, after SC formation is completed (at least in the pro-oocytes, see below), suggesting meiotic DSBs form after SC formation. The foci then disappear before region 3 (late pachytene), which is slightly earlier than late RNs disappear, which persist into region 3 and sometimes the vitellarium (Carpenter 1979b). These results indicate that all DSBs may have been repaired by the end of the germarium (region 3) but unresolved recombination intermediates may persist later in prophase.

Two genes have been identified that have a direct role in DSB formation. *Drosophila* mutants *mei-W68* and *mei-P22* lack all meiotic recombination. These mutants also lack γ -His2Av staining and suppress developmental defects associated with DSB repair defective mutants (see below). The crossover formation defects in these mutants are partially rescued by irradiation with X-rays, consistent with the other evidence for the lack of DSB formation in these mutants.

Immunofluorescence studies show that MEI-P22 localizes to foci associated with meiotic chromosomes (Liu et al. 2002). MEI-P22 expression is restricted to early pachytene, consistent with the need to restrict DSB formation in prophase. MEI-P22 foci first appear in earlier stage oocytes than γ -His2Av foci, but the two appear in similar numbers and often colocalize (Mehrotra and McKim 2006). The appearance or removal of MEI-P22 occurs independently of DSB formation, which suggests that MEI-P22 localization is not related to repair or response to DSBs (Liu et al. 2002). MEI-P22 foci depend on the structure of the chromosomes, since they are not observed in *c(3)G* mutants and their numbers are reduced in *c(2)M* mutants.

4.2

The SC Promotes Meiotic DSB Formation in Oocytes

DSB formation also appears to be affected by *C(3)G* and *C(2)M*. The number of γ -His2Av foci is reduced to 20% of wild-type level in *c(3)G* mutants and 40% of the wild type in *c(2)M* mutants. The reduced number of γ -His2Av foci in these mutants suggests that SC formation and localization of *C(3)G* are not absolutely required for DSB formation. The fact that *C(2)M* and *C(3)G* are required for crossover generation indicates that SC components are involved in crossover directed repair of DSBs. The timely disappearance of the γ -His2Av foci in *c(3)G* and *c(2)M* mutants also suggests that components of the SC are not required for DSB repair (Mehrotra and McKim 2006). Other meiotic chromosome proteins, such as ORD (Webber et al. 2004) and PDS5 (Mehrotra and McKim 2006), are not required for DSB formation.

A comparison of γ -His2Av staining between oocytes and nurse cells (Fig. 2) has shown that the requirement of *C(3)G* for DSB formation is specific to oocytes. Although γ -His2Av and MEI-P22 foci appear in the pro-oocyte after the completion of the SC, both types of foci are also observed in the nurse cells with little or no SC formation. Similarly, SC defective mutants have little or no effect on the number of γ -His2Av foci in nurse cells (Mehrotra and McKim 2006). These results suggest that the dependence of DSB formation on the SC is specific to the oocytes. Since the SC does not affect phosphorylation of His2Av (Mehrotra and McKim 2006), it is simplest to propose that some SC components have a regulatory role in DSB formation and this regulation occurs at the level of DSB formation. It is possible, however, that some γ -His2Av foci have a shorter lifetime due to alternative modes of DSB repair being employed in the absence of the SC. The most important regulator of DSB formation may be timing, at some specific point after entering meiotic prophase. Indeed, the timing of γ -His2Av foci appearance in the pro-oocytes and nurse cells follows similar dynamics. That is, foci appear at the same time in pro-oocytes and their sister nurse cells within the same cyst.

4.3

SC is Not Sufficient for DSB Formation

Approximately 30% of each *Drosophila* autosome and 45% of the X chromosome is composed of centric heterochromatin. These vast regions of mostly repetitive DNA with only the occasional gene rarely, if ever, experience a meiotic crossover, although they can be induced by X-rays (Roberts 1969). In addition to highly repeated DNA, these regions are characterized by the enrichment of some epigenetic modifications, such as methylation of histone H3 at lysine 9 and localization of HP1 protein (Eissenberg and Elgin 2000). Electron microscopy (EM) studies (Carpenter 1975) or immunostaining at the light microscopy level (Mehrotra and McKim 2006) suggest that SC assembles in the heterochromatin. In comparison to euchromatic SC, Carpenter (1975) described the heterochromatic SC as being relatively amorphous, thinner, more flexible, and enclosed by condensed chromatin. More recently, it has been found that γ -His2Av foci are absent in these regions, suggesting that DSB formation does not occur in the heterochromatin (Mehrotra and McKim 2006). The absence of DSB formation could be related to the differences in SC and chromatin structure, although it is also possible that nuclear position plays an important role in DSB formation, independent of the ability to form SC.

5

From DSB Repair to Crossover Formation

Genetic and cytological lines of evidence show that the majority of DSBs do not become crossovers. Studies at the *rosy* locus originally suggested that only 20% of intragenic recombinants are associated with crossovers (Hilliker et al. 1988). However, the *ry* locus is in a part of the chromosome that experiences fewer crossovers per Mb than the genome average, which could reflect a lower crossover/noncrossover ratio than the genome average (McKim et al. 2002). A more accurate genome-wide estimate comes from comparing the number of DSBs (assuming each becomes a noncrossover or crossover) to the number of crossovers in the genome (1.3 per arm or 6.5 per genome). One estimate of DSB number in *Drosophila* comes from γ -His2Av foci (20–24 per genome, see Sect. 5.1), which gives an estimate of one in 3–3.7 recombinants associated with a crossover (Mehrotra and McKim 2006).

There are several models for DSB repair but the simplest is that the outcome of noncrossover or crossover depends on which strands in a double Holliday junction are cut (Szostak et al. 1983). Additional models for DSB repair have been proposed that could easily incorporate genetic controls because they provide a mechanism to produce gene conversions but not crossovers (Paques and Haber 1999) (Fig. 4). One model asserts that cleavage of both Holliday junctions is orchestrated to result in a crossover, whereas

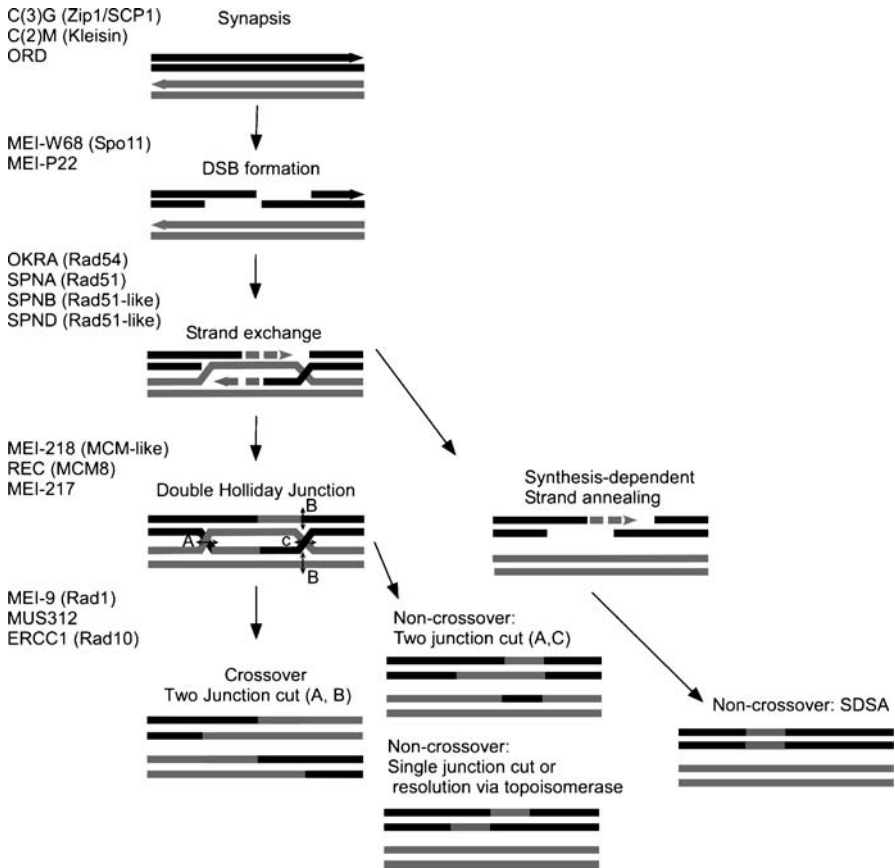


Fig. 4 Meiotic recombination pathway and the genes required at each step. The DSB repair model utilizing the resolution of the double Holliday junction intermediate can result in both crossovers and noncrossovers. The Holliday junction-independent repair model is the synthesis-dependent strand annealing (SDSA) mechanism and results in only a non-crossover. The proteins required for synapsis, like C(3)G, are also required for crossing over. Precondition genes like *mei-218* could be required earlier than DSB repair genes, since when the decision to become a crossover occurs is not known and could be early in the pathway. For example, there could be a branch before the double Holliday junction leading to SDSA instead. In this model, progressing down the double Holliday junction branch requires MEI-218

a noncrossover results from alternative resolution pathways such as (1) resolution of one Holliday junction followed by branch migration of the other junction past the nicks, or (2) branch migration of both junctions toward each other and then decatenation with topoisomerase. An alternative model suggests that noncrossovers may result from a Holliday junction-independent pathway, such as synthesis-dependent strand annealing (SDSA). In this model a double Holliday junction does not form, rather strand invasion is followed

by DNA synthesis primed by one or both broken ends and then reannealing of these strands. Any of these alternative models could explain the crossover-specific phenotypes of mutants like *mei-218* and *mei-9* in *Drosophila* (see Sect. 5.2). While physical evidence is lacking, genetic evidence suggests that a DSB repair model involving a double Holliday junction does apply to meiotic recombination in *Drosophila* (Radford et al. 2007a,b). The decision to form a crossover in *Saccharomyces cerevisiae* has been proposed to be established early in the pathway, at the stage of strand invasion or prior to the DSB formation (Bishop and Zickler 2004; Borner et al. 2004). This may also be true in *Drosophila* (E. Joyce and K. McKim, unpublished data, see Concluding Summary).

5.1

DSB Repair Proteins

Like most organisms, proteins in the Rad52 epistasis group are required for meiotic DSB repair. These include strand exchange proteins such as Rad51 and other repair proteins such as Rad54. The orthologs of Rad51 and Rad54 are encoded by the genes *spn-A* (Staeva-Vieira et al. 2003) and *okr* (Ghabrial et al. 1998; Kooistra et al. 1997), respectively. In *Drosophila*, mutations in these genes cause a persistence of γ -His2Av foci into late pachytene stages, consistent with a block in the repair of meiotic DSBs (Jang et al. 2003; Mehrotra and McKim 2006). This may be the cause of the *spindle* phenotype of these mutants, which refers to their effect on the shape of the mature oocyte. These mutants have dorsal-ventral patterning defects in the oocytes and are sterile, which appears to be a secondary consequence of activating the DSB repair checkpoint during prophase (Ghabrial et al. 1998; Ghabrial and Schupbach 1999). This hypothesis has been supported by the observation that the patterning defects in *spn-B* and *okr* mutants are suppressed by *mei-41* mutations (Ghabrial and Schupbach 1999). MEI-41 is the *Drosophila* ortholog of the Rad3/ATR DNA-dependent protein kinase involved in DSB-mediated checkpoint response. Furthermore, the sterile phenotype in the DSB repair mutants is suppressed by *mei-W68* and *mei-P22* mutants, which are defective in DSB formation.

In addition, the *Drosophila* genome encodes additional Rad51 paralogs, at least two of which, *spn-B* (Ghabrial et al. 1998) and *spn-D* (Abdu et al. 2003), are also required for meiotic DSB repair. Despite this plethora of paralogs, there is no clear ortholog of DMC1, the meiosis specific Rad51. In addition, there are no orthologs of two other proteins involved in meiotic DSB repair of other organisms, Hop2 and Mnd1. All three proteins could be absent because there is no requirement of DSBs for synapsis (Ramesh et al. 2005). In addition, some functions of DMC1, such as for interhomolog interactions (Schwacha and Kleckner 1997), may be carried out by the other Rad51 paralogs. Finally, the *spn-C* gene encodes the *Drosophila* ortholog of human HEL308, a single-

stranded DNA-dependent ATPase and DNA helicase of unknown function. Like the Rad51 and Rad54 orthologs, SPN-C is required for meiotic DSB repair and progression (Laurencon et al. 2004; McCaffrey et al. 2006).

These five known genes required for meiotic DSB repair have a strikingly similar effect on the appearance and duration of γ -His2Av foci (Mehrotra and McKim 2006). First, a similar number of foci persist into late pachytene (past region 3) in each of these mutants. Since it is simplest to conclude that no DSBs are repaired in any of these repair mutants, the number of foci (20–24) is probably an accurate estimate of the total number of DSBs induced early in pachytene. Second, the accumulation of γ -His2Av foci in response to meiotically induced DSBs, which normally occurs in region 2a, is delayed in *spn-B*, *spn-D*, and *spn-A* mutants. These mutants also show a delayed γ -His2Av response to X-ray-induced breaks, suggesting these genes are required for a rapid response to DSBs. A role in responding to DSBs is discussed more in the Concluding Summary.

5.2

Establishing Crossover Sites

It was suggested above that the DSB repair pathway diverges into crossover and noncrossover pathways. Three groups of genes may have a role in the crossover-specific part of the pathway based on the observation that these genes are required for crossover formation but not DSB formation or repair. The first group comprises SC proteins. Although DSBs are formed in *c(3)G* mutants (see Sect. 4.2), no crossovers are observed, suggesting the SC is required for crossing over (Hall 1972). Unlike *mei-W68* or *mei-P22* mutants, the crossover defect in *c(3)G* mutants is not rescued by X-ray-induced breaks, suggesting that the generation of a DSB in the context of SC formation is required for generating crossovers (Bhagat et al. 2004). *C(2)M* is also required for crossing over, perhaps through its effects on *C(3)G*. Similarly, *ORD* is also required for most crossover formation and is required for assembly of the SC (see Sect. 3.2) (Bickel et al. 1997; Webber et al. 2004).

Members of the second and third groups are required for crossing over, but there is no effect on SC formation. Examples of these two groups are *mei-218* and *mei-9* mutants, which eliminate 90% of the crossovers, but not gene conversions (Carpenter 1982, 1984). This suggests that these mutants are able to initiate the recombination pathway but have defects in the repair pathway that leads to crossover formation. Consistent with this notion, X-rays do not induce crossovers in *mei-218* mutants (Bhagat et al. 2004). These are candidates for crossover-specific genes since they are not required for SC formation or DSB repair. They are further divided into two types: the *precondition* and the *exchange* genes.

These genes have primarily been defined by their effect on crossover distribution. Exchange mutants reduce crossing over uniformly along the chromo-

some. In contrast, precondition mutants reduce crossing over nonuniformly. The reductions in crossing over are less severe near the centromeres, resulting in a change of crossover distribution that more closely resemble the physical map. Since precondition mutants affect the distribution of crossovers, these genes have been proposed to determine which sites become crossovers (Carpenter and Sandler 1974; Sandler et al. 1968). The problem with this definition is that a diverse array of mutants have an effect on the distribution of crossovers (Bhagat et al. 2004). These include mutations in the genes encoding components of the SC: *c(3)G* and *c(2)M*, hypomorphic alleles of genes required for DSB formation such as *mei-W68* and *mei-P22*, and mutations in DSB repair genes like *spn-B* and *okr*. What separates the precondition genes from the rest, however, is their specificity. Among mutants that affect crossover distribution, only *mei-218*, *mei-217*, and *rec* affect crossovers. SC formation occurs normally, DSBs are generated, and then they are repaired without activating a DSB repair checkpoint.

Carpenter observed fewer late RNs in *mei-218* mutants than in the wild type (Carpenter 1979a), down to approximately 16% of the wild type. About half of the late nodules that were observed in *mei-218* mutants had abnormal morphology (Carpenter 1979a). These results are consistent with a function for MEI-218 in determining crossover sites. An unexplained observation, however, is that there are approximately 30–40% of the normal number of early RNs in *mei-218* mutants compared to the wild type (Carpenter 1979a, 1989). As noted by Carpenter, this does not mean that there are fewer early nodules since they were also observed over a broader range of stages. It is possible that the early RNs are less stable and present for a shorter period of time in *mei-218* mutants. Thus, the observation of fewer early RNs does not necessarily contrast with the observation that *mei-218* mutants do not have reduced frequency of noncrossover recombinants. This observation does raise questions about when MEI-218 functions in the pathway to generate crossovers (see Concluding Summary). Unfortunately, the RN phenotype is rarely ascertained, for example, in the other precondition mutants.

Both MEI-218 (McKim et al. 1996) and REC (Blanton et al. 2005) are related to the MCM family of proteins. REC is the *Drosophila* ortholog of MCM8 with a complete MCM domain including residues for ATP hydrolysis. MEI-218 is more distantly related with only a portion of the MCM domain. The link of precondition genes to MCM proteins has been reinforced with the discovery of an *mcm5* allele with similar phenotypes to *mei-218* (R.S. Hawley, personal communication). How this homology relates to specifying crossovers, and if this involves events during DNA replication, is not known. A reasonable hypothesis is that precondition genes like *mei-218* and *rec* are required for the repair of a DSB to follow a crossover rather than a noncrossover pathway. Based on epistasis to *mei-9* mutants (see Sect. 5.4), the precondition genes may be required to promote the formation of a DSB repair intermediate capable of becoming a crossover (Bhagat et al. 2004). There are clues, such as

the effect on early nodules, that *mei-218* may function early in the DSB repair pathway. This is consistent with models in which the meiotic DSB repair pathway separates early into crossover or noncrossover branches (Fig. 4).

5.3

Nonspecific Crossover Defective Mutants

Some mutants have rather pleiotropic effects on *Drosophila* meiosis. A recent example is *mei-352* (also known as *klp3A*), which alters the distribution of crossovers without altering the overall frequency of crossovers. MEI-352 is a kinesin-like protein in the Kinesin-4 (chromokinesin) family. The mechanism by which MEI-352 is involved in the distribution or specification of exchanges is not yet understood. Based on evidence suggesting that kinesin motor proteins contribute to chromosome organization in other systems, it is possible that the abnormal distribution of exchanges in *mei-352* mutants could be due to defective organization of homologous sequences during meiotic prophase (Page and Hawley 2005). However, less direct mechanisms may be at work since microtubule organization is important for oocyte differentiation. Indeed, mutations in genes required for oocyte differentiation, such as *mei-P26* (Page et al. 2000) and *Sxl* (Bopp et al. 1999), reduce the frequency and alter the distribution of crossovers, similar to *mei-218* and *rec*. What makes *mei-218* and *rec* different is the specificity of their mutant phenotype for crossover formation.

5.4

The Exchange Reaction: The Paradox in Making Crossovers

The only mutations that do not affect crossover distribution are members of the exchange class such as *mei-9* and *mus312* (Carpenter and Sandler 1974; Yildiz et al. 2002). These genes have been proposed to be directly involved in the crossover resolution process. Several epistasis experiments suggest that *mei-218* functions prior to exchange genes like *mei-9* in the recombination pathway. Unlike *mei-218* mutants, *mei-9* mutants have no effect on late RNs (Carpenter 1979a). With respect to the crossover distribution phenotype, *mei-218* mutants are epistatic to *mei-9* (Sekelsky et al. 1995). *mei-9* mutants exhibit high rates of postmeiotic segregation (PMS) at the *ry* locus, whereas PMS is not observed in *mei-218* mutants or in *mei-9 mei-218* double mutants (Bhagat et al. 2004). PMS is the phenomenon where mismatches formed during DSB repair are not repaired, causing them to segregate at the first division following meiosis.¹ It is not clear why *mei-9* mutants fail to repair these mismatches, although it might be related to the failure to nick Holliday junctions

¹ PMS leads to mosaic progeny, with a mixture of *ry*⁺ and *ry*⁻ tissue that can be detected by visual examination or allele-specific PCR.

(Radford et al. 2007a). These results are consistent with the model that pre-condition genes such as *mei-218* are required to determine which DSBs will be repaired as crossovers, while exchange genes such as *mei-9* are required for the resolution reaction. The PMS experiments suggest that MEI-218 could determine which DSBs become crossovers by promoting the formation a DSB repair intermediate which can be resolved into a crossover.

mei-9 encodes an ortholog of XPF/Rad1, which as a dimer with ERCC1/Rad10 is an endonuclease involved in excision repair (Sekelsky et al. 1995). The *Drosophila* homolog of ERCC1 interacts with MEI-9 and is also required for meiotic crossing over (Radford et al. 2005). Interestingly, unlike their effects on excision repair, *mei-9* mutants have more severe effects on meiotic crossing over than *Ercc1* mutants, suggesting that MEI-9 may function in the absence of ERCC1. Indeed, MEI-9 interacts with another protein, MUS312. *mus312* mutants also show similar crossover defective phenotypes as observed in *mei-9* mutants. MUS312 also has a role in somatic repair and has been shown to interact with MEI-9 in a yeast two-hybrid assay (Yildiz et al. 2002). Thus, a protein complex including MEI-9, MUS312, and ERCC1 may be involved in the resolution process that generates crossovers. That this complex probably has endonuclease activity is consistent with a role in resolving a recombination intermediate such as a Holliday junction into crossover, although such an activity has not yet been demonstrated.

What is surprising about the exchange class is that a role for proteins like MEI-9 and ERCC1 in meiotic crossover formation is not conserved. No other organism is known to use MEI-9 and ERCC1 for making crossovers. In addition, *Drosophila* does not require Mlh1 for crossovers (E. Joyce and K. McKim, unpublished results) and it lacks the genes encoding Msh4 or Msh5, which are meiosis-specific MutS paralogs. All three proteins are required for crossing over in many other organisms (Hoffmann and Borts 2004). The paradox is that other indicators show that crossover formation in *Drosophila* is the outcome of a conserved process. For example, like most organisms, all crossovers in *Drosophila* require the SC (Page and Hawley 2004). In addition, several conserved DSB repair proteins are used by *Drosophila* and most other organisms as part of the pathway for generating crossovers. These include a complex of DSB repair proteins in mammals with *Drosophila* orthologs, Rad51C (SPN-D) and XRCC3 (SPN-B), which have been proposed to be part of a Holliday junction resolvase (Liu et al. 2004). A similar situation may exist in *Schizosaccharomyces pombe*, where a relative of the Mei-9/ERCC1 complex, Mus81/Eme1, is involved in crossover formation (Osman et al. 2003).

It could be that the last step of the crossover pathway is less evolutionarily constrained than earlier steps like DSB formation and repair. Under the right circumstances, several proteins (or complexes) may be capable of cleaving a Holliday junction. Or the resolvase complex could contain conserved components (SPN-D/Rad51C and SPN-B/XRCC3) and less conserved components

(MEI-9/XPF). Another variable is that alternative mechanisms to generate crossovers, utilizing an unligated Holliday junction (Hollingsworth and Brill 2004) or a single Holliday junction (Cromie et al. 2006) have been proposed in *S. pombe* (see G. Cromie and G.R. Smith, this SERIES). Thus, specifying crossover sites may be a more highly conserved process than the resolution.

6

Crossover Control at the Chromosomal Level

6.1

Chromosome Structure and the Distribution of Crossovers

The frequency of crossovers per unit of physical distance increases the farther the genetic interval is from the centromere (McKim et al. 2002). The existing evidence suggests that this distribution does not reflect the pattern of DSB formation. One data set comes from the distribution of early RNs, which appear uniform along each chromosome arm (Carpenter 1979b). The other relevant data set is that the frequency of gene conversion is the same at the *rosy* locus (Hilliker et al. 1988), which experiences crossovers, and *maroon-like*, which is so close to the centric heterochromatin that it rarely experiences crossovers (Smith et al. 1970). Nonetheless, both these data sets represent only a small sampling of meiotic events, and this is an area that would benefit from more extensive analysis of DSB distribution.

These observations also raise the question of whether there are recombination “hotspots” present in the fly genome. As originally described in *S. cerevisiae* (Lichten and Goldman 1995) but also observed in other fungi and mammals (Kauppi et al. 2004; see C. May, T. Slingsby and A.J. Jeffreys, this BOOK), certain regions in the genome can experience exceptionally high recombination frequencies. No such sites have been mapped in *Drosophila* so far.

6.2

Role of Boundary Sites and Chromosome Domains in Crossover Formation

As described above (Sect. 3.3), translocation heterozygosity suppresses crossing over within an interval defined by specific (boundary) sites (Fig. 3). Since crossover suppression is not associated with defects in homolog pairing (Sherizen et al. 2005) or DSB formation (Gong et al. 2005), translocation heterozygosity may affect how DSBs are repaired, perhaps similar to the way a precondition mutant allows noncrossovers but not crossovers. There are no physical impediments to generating or recovering a crossover in a translocation heterozygote, therefore we have proposed the effect is on chromosome structure (Sherizen et al. 2005).

Translocation heterozygosity causes a break in the chromosome axis, and this may be the cause of crossover suppression. A model for how this occurs is based on the idea that specialized (*boundary*) sites are required to establish or maintain domains of chromosome structure. Continuity of the chromosome axis between boundary sites is necessary for these domains to function or be organized correctly, and this is a precondition for DSBs to be repaired as crossovers (Fig. 3). The nature of these domains is not known, but understanding this may provide insight into how higher order chromosome structure has a role in crossover formation. For example, these domains could establish the mechanical properties of the chromosomes required for crossovers to form (Kleckner et al. 2004). The boundary sites could play an active role, such as to promote assembly important axial components. Alternatively, they could have a passive role, such as in maintaining the chromosome structure within a domain but not being involved in their establishment.

The SC may be an example of a structure that may need to be continuous for crossover formation to occur. While numerous patches of chromosome-associated C(3)G accumulate in *c(2)M* mutants, crossing over is strongly reduced (Manheim and McKim 2003), suggesting that continuity of SC is required for crossing over, not just the presence of SC proteins. An interaction between boundary sites and the SC is a possibility, but currently there is no evidence that these sites have a role in SC assembly.

6.3

Ensuring at Least One Crossover

The distribution of crossovers is nonrandom along and between chromosomes. One of the mechanisms that have been proposed to play a key role in crossover control is crossover interference. Because double crossovers are much less frequent than predicted assuming a random distribution of crossovers, crossover interference has been suggested to regulate reciprocal recombination such that chiasmata are distributed over the entire chromosome and the genome, and also that each pair of homologs does not recombine excessively.

Two observations suggest that *Drosophila* lacks an effective system to ensure at least one crossover per chromosome. First, when crossing over is reduced by a translocation in one genetic interval of a chromosome, this did not result in a compensatory increase in crossing over in other intervals (Sherizen et al. 2005). This suggests that crossover suppression by translocations does not activate a system that is involved in ensuring at least one crossover per arm. This does appear to occur when chromosomes are rearranged in *C. elegans* (Hillers and Villeneuve 2003; McKim et al. 1993) and *S. cerevisiae* (Kaback et al. 1992, 1999).

Second, the probability that a DSB becomes a crossover is not easily increased. As noted above, we have estimated that there are about three DSBs

for every crossover. If the frequency of DSBs is reduced, however, the probability that the remaining DSBs can become a crossover is not dramatically increased (Mehrotra and McKim 2006). This contrasts with results in *S. cerevisiae* (e.g., Martini et al. 2006). Thus, *Drosophila* lacks a system which can readily detect a low number of DSBs, and may be a case where there is robust interference but a weak obligate crossover mechanism.

7

Concluding Summary

Drosophila is unusual in that the SC forms in the absence of DSBs. *Drosophila* is also a little unusual in how it forms crossovers, with a complex including the endonuclease MEI-9/ERCC1. But the steps in between have many features in common with other organisms. Some of the most intriguing questions center on how DSB sites are selected and how a subset of these sites are selected to become crossovers. Approximately 20–24 DSBs, or four to five per chromosome arm, are generated each meiosis. With the existing tools it should be possible to determine the distribution of DSBs along a chromosome arm and gain insight into how these sites are selected.

Important insights may emerge from studying the chromosomal context in which the DSBs are made and how that relates to repair and crossover formation. We have suggested that crossover formation depends on the structure of the chromosome within long domains. The boundaries of these domains can be genetically mapped and the relevant sequences need to be cloned. At that point, they can be compared to “pairing centers” in *C. elegans*, which have a role in pairing and synapsis and are associated with the nuclear envelope (Phillips and Dernburg 2006; Phillips et al. 2005).

Both the SC and the proposed chromosomal domains involved in crossover formation assemble independently of the DSB repair pathway. Given that the decision to form a crossover in *S. cerevisiae* occurs at strand invasion or prior to formation of the DSB (Bishop and Zickler 2004; Borner et al. 2004), is there any other evidence in *Drosophila* of steps in the crossover pathway that occur early in the pathway or independent of DSBs? The γ -His2Av response to DSBs depends on DSB repair proteins in early pachytene cells but not other germline cells (Sect. 5.1). This suggests that certain conditions may exist before or shortly after DSB formation that influence how the oocyte responds to a DSB. Perhaps the repair machinery is already assembled before the DSB is formed, possibly with fine-tuning to control whether a crossover will be formed. Another clue is that DSB repair (McCaffrey et al. 2006) and exchange mutants have DSB-independent phenotypes which could reflect a delay in prophase progression (E. Joyce, and K. McKim, unpublished results). In *C. elegans* and *S. cerevisiae*, Pch2, an AAA-ATPase, is required for a DSB-independent surveillance mechanism (Bhalla and Dernburg 2005;

Wu and Burgess 2006) and a similar mechanism may exist in mammals (Di Giacomo et al. 2005). Further studies will determine if Pch2 monitors a DSB-independent process in *Drosophila* and what is being monitored. This may provide clues as to what must be done to establish a crossover site.

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Synaptic and Recombination Nodules in Mammals: Structural Continuity with Shifting Protein Composition

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Abstract The processes of meiotic recombination and synapsis between homologous chromosomes are intimately related, although their interdependence may vary with different organisms. In many species, electron-dense nodules are observed in association with pairing forks and/or fully established synaptonemal complexes. However, there are significant differences in the time of appearance, the relative number of early vs. late nodules, and even in the size and shape of nodules between species. This chapter focuses on the formation of synaptic and recombination nodules in mammals—primarily in mice and humans. In particular, the chapter reviews the transient formation of foci containing various recombination proteins, such as Rad51/DMC1, BRCA1/2, ATR, MSH4/5 and others—in correlation with recombination nodules. Despite the assumption that both the protein components and the basic functions of nodules are evolutionarily conserved, the evidence summarized here suggests that this may not be the case.

Abbreviations

AN axial nodule
AE axial element
cM centimorgan
DSB double-strand break
RN recombination nodule
SC synaptonemal complex
SyN synaptic nodule

1

A Note on Nomenclature

The term “recombination nodule” (RN) was coined by Carpenter (1975) and rapidly became the accepted term for electron-dense nodular structures observed during meiotic prophase. The number of these structures tended to show a correlation with the expected number of crossovers, whether or not that expectation was based on chiasmata counts or on genetic linkage maps. There is no universally accepted term for the second type of nodule observed earlier in prophase than RNs. Referring to these as “early RNs” is confusing, since the term implies that all early nodules will eventually become RNs and

be involved in (reciprocal) recombination. While the phrase “early nodule”, or “EN” is temporally appropriate, it gives no hint as to the likely function of these structures. The nomenclature “meiotic nodule” is also accurate, but again non-informative. Moreover, these nodules can be further subdivided into two categories: those found on asynapsed axes and those found on synapsed axes. In this review, the term “axial nodule” or AN will be used to refer to nodules on asynapsed axial elements (AEs) and the term “synaptic nodules” or SyNs will be used to refer to nodules located along the synaptonemal complex (SC). The term “synaptic nodule” or SyN has been chosen to better reflect the role that these early nodules are thought to play in the check for homology that accompanies synapsis. Moens et al. (2002) have suggested the terms “early nodule” and “transitional nodule” to reflect changes in protein composition during pachynema. There are many changes in protein composition, discussed below, within early nodules (both ANs and SyNs) during early meiotic prophase. These can include addition or accumulation of proteins to ANs if homologs do not synapse. If homologs do synapse, there is both assembly (addition of proteins) and disassembly (loss of proteins) in these SyNs as the bivalents proceed through prophase. Therefore, it seems more appropriate to use nomenclature that focuses on the types of structures originally observed by electron microscopy.

2

Historical Background

2.1

Recombination Nodules

In a classic paper, Adelaide Carpenter (1975) observed and described an electron-dense structure in *Drosophila* oocytes that she termed a “recombination nodule”. By serial sectioning an entire germarium (453 serial sections, with the loss of but one section), she was able to accurately determine the meiotic stage of each oocyte and follow the appearance and disappearance of these electron-dense, roughly spherical structures. She described them as situated “adjacent to the central element and spanning the width of the SC”, therefore located “between and adjacent to the chromatin of the paired homologs”. She reported the following correlations between her RN observations and the existing genetic data: (1) The maximum number of nodules in a single nucleus was five, a number in agreement with the expected 5.6 exchanges per nucleus predicted from the genetic map length. (2) There were no nodules in heterochromatin where crossovers do not occur. (3) Most RNs occurred in the middle and distal portions of the euchromatic portions of bivalent arms, a pattern similar to the calculated crossover distribution. (4) Most bivalent arms had one nodule; only a few had two. Those arms with

one crossover tended to have a nodule in the middle of the arm (consistent with genetic data on single-exchanges), while the only two bivalents with two had one proximal and one distal, these being located quite a distance from one another, consistent with models of crossover interference. For a full discussion, see Carpenter (1975).

Carpenter (1979b) did not find RNs on SCs of oocytes in zygonema or early pachynema as judged by their position in the germarium. The maximum number of RNs occurred in mid-pachytene oocytes with the number decreasing in older oocytes. Therefore, she concluded that crossovers in *Drosophila* (primarily) occurred during mid-pachynema (see also S. Mehrotra, S. Hawley and K. McKim, in this BOOK).

Confirmation of RNs in meiotic prophase nuclei of other species rapidly followed with reports of their occurrence in fungi such as *Sordaria* (Zickler 1977), in mammals including humans (Rasmussen and Holm 1978), and in plants such as rye (Abirached-Darmency et al. 1983).

2.2

Synaptic Nodules

As already alluded to above, it soon became clear that not all nodules are RNs. In the Ascomycete *Sordaria macrospora* Zickler (1977) observed two sizes of nodules on the seven synapsed SCs. Large electron-dense nodules were present from zygonema until diplonema and appeared oval when viewed in longitudinal sections (60×100 nm) and more spherical when observed in cross section (70 nm). Small nodules appeared earlier in zygonema, disappeared during pachynema, and were less electron-dense and more spherical, varying in size between 40 and 60 nm (Zickler 1977). The large nodules closely corresponded to the number of chiasmata, supporting the supposition that large nodules are RNs. Consistent with this conclusion, short stretches of the SC persisted at diplonema at sites where homologs are held together by chiasmata and most of these persistent SC stretches contain large nodules (Zickler 1977). In a later study, Zickler et al. (1992) found a good correspondence between the number and distribution of these large nodules (RNs) and genetic map data.

However, the small nodules had a different temporal profile. Although both types were present on SCs in zygonema, the small nodules were detectable as soon as SC formation began, often occurring at pairing forks where axial elements (AEs) converged at the forming SC (Zickler et al. 1992). By mid-pachynema they were no longer detectable. Although small nodules were less dense than the RNs and their shape less regular, the total number of early nodules observed in zygonema of *Sordaria* never exceeded the number of RNs (Zickler et al. 1992). While the number of these early nodules decreased as zygonema progressed, the number of RNs increased then remained constant throughout pachynema into diplonema (Zickler 1977; Zickler et al. 1992). The

parallel increase in RNs with the decrease in SyNs suggests conversion of all SyNs to RNs in this species.

Carpenter (1979b) also detected a second type of nodule in *Drosophila* where the nodules were more ellipsoidal than RNs and appeared earlier in pachytene oocytes. She observed none in zygonema, a very short stage in *Drosophila* since this species has somatic pairing which results in prealignment of homologs. In early pachynema, oocytes had both ellipsoidal and spherical nodules in the same nucleus (Carpenter 1979b). Although the maximum number of ellipsoidal nodules she observed per nucleus (9) was slightly higher than the maximum number of RNs observed per nucleus (6), there was, however, a difference in their distribution. A large proportion of bivalent arms had three SyNs compared to the maximum of two RNs per arm, leading her to conclude that there were fewer biological constraints on these ellipsoidal nodules than on RNs—i.e., they did not appear to be subject to crossover interference. She later proposed that these early nodules were involved in gene conversion events instead of crossovers and argued against the concept of conversion of one type of nodule to another (Carpenter 1987) (see also S. Mehrotra, S. Hawley and K. McKim, in this BOOK).

Observations in higher plants afford yet another view of early nodules. It is important to recognize that the distinction between ANs and SyNs in these early EM studies is based only on where they occur. If they are on AEs, they are here called ANs; if they are on SCs, they are here called SyNs. It is highly likely that ANs become SyNs once homologs have synapsed. In 2D spreads of zygotene microsporocyte of *Lycopersicon esculentum* (tomato), Stack and Anderson (1986) observed numerous early nodules associated with the newly formed SCs. These nodules varied in size from small and round (50×50 nm) to large and ellipsoidal (250×290 nm). Although they noted some early nodules (ANs) on asynapsed AEs, the vast majority were SyNs, since they were located on the newly synapsed SCs. In maize they noted a high number of SyNs at synaptic forks (Stack and Anderson 2002). Given that the frequency of early nodules per unit length did not change during zygonema, they concluded that early nodules (SyNs) do not continue to assemble on SCs after homologs have synapsed. This localization plus the differences in size and shape of these nodules led them to suggest that early nodules were preferentially assembled at synaptic forks. Based on the observed average of 1.41 nodule per μm of SC, they estimated 358 early nodules (SyNs) per nucleus. The number of these nodules dropped to 0.33 per μm by early pachynema and had virtually disappeared by late pachynema. Since the average number of RNs per nucleus is 20.5 in tomato they estimated the ratio of SyNs to RNs at around 16 : 1 (Stack and Anderson 1986).

Albini and Jones (1987) examined synapsis in *Allium cepa* and *A. fistulosum*, two species of onion, and noted extensive AE formation before SC formation. They observed ANs, which they called “meiotic nodules”, at many sites along the asynapsed AEs as well as on the newly formed SCs during

zygonema. In addition, they noted multiple synaptic initiation (association) sites characterized by alternating convergences and divergences of the AE and often observed elongated (bar-shaped) nodules at these convergences. Similar “bridging” of early nodules between homologous AEs was also reported in *Psilotum nudum* (Anderson and Stack 1988). In some cases of aligned, but diverged AEs in *Allium Albinii* and Jones (1987) observed ANs at *corresponding* sites along the homologous AEs, as though each pair of homologs had its own set of ANs and potential synaptic initiation sites. When the nodules did not straddle the converging axes, they appeared to be spherical and ranged in size from 100–170 nm (average 140). As was the case in tomato, the number of SyNs on the SCs far exceeded the expected number of RNs or chiasmata. However, in *Allium*, the SyNs disappeared from the SCs by the end of zygonema.

The general conclusion of most of these zygotene/early pachytene studies was that SyNs were involved in a molecular check for homology associated with synapsis (Albini and Jones 1987; Stack and Anderson 1986; Zickler 1977). Both Stack and Anderson (1986) and Albini and Jones (1987) suggested that a subset of SyNs might become RNs.

2.3

Nodules in Mammals

Despite the close correlation between the number of RNs and reported number of crossovers in *Drosophila*, not all species exhibit such a close correspondence. The most notable discrepancy is *Homo sapiens*. Rasmussen and Holm (1978) observed small (minimum diameter 30 nm) spherical “nodules” in zygotene and late “nodules” or electron-dense “bars” in pachytene spermatocytes. Although they never observed as many bars in any pachytene nucleus as the reported number (50) of chiasmata (Hultén 1974), they suggested that the “bars” were RNs. Whether some “nodules” in this study were also RNs remains a subject for debate. However, it is evident that many of the “excess” nodules in the early pachytene nuclei were SyNs (discussed below). By early pachynema when all autosomes are completely synapsed, the observed number of “nodules” (not bars) had fallen to an estimated mean of 75 nodules per nucleus, a number 50% higher than the reported number of chiasmata (Rasmussen and Holm 1984). The earliest pachytene nuclei had no bars, but mid-pachytene nuclei had an average of 35. After mid-pachynema the number of bars steadily decreased until by late pachynema they observed an average of only four bars per nucleus (Rasmussen and Holm 1984). Since Hultén had reported 50 chiasmata per diakinesis nucleus in human spermatocytes, the observed number of bars at any stage was insufficient to account for the predicted number of crossovers. They therefore assumed that some structures they had classified as nodules were being converted to bars and that there was a turnover of both nodules and bars during pachynema (Ras-

mussen and Holm 1984). As will be discussed below, when antibodies to RN components are used on human pachytene spermatocytes, the numbers closely correspond to the estimated number of crossovers, suggesting that it is unlikely the problem of distinguishing between bars (RNs) and nodules (SyNs) was due to asynchrony. This study makes no mention of nodules on asynapsed axes.

A similar deficiency of nodules plus bars compared to chiasmata counts was observed in sectioned material of human oocytes (Bojko 1985). She found a mean of 68 nodules at late zygonema. However, she also reported a mean of 30 nodules at early diplonema. These results are difficult to interpret since there is no report in other species of SyNs (nodules) persisting until diplonema. An alternate interpretation is that nodules and bars are hard to differentiate in human oocytes and that these "nodules" were misclassified and should have been recorded as bars. She reported that the number of bars increased from a mean of six at late zygonema to a mean of 17 at late pachynema. Yet the total number of nodules and bars per nucleus (59 ± 5) in mid-pachynema, exceeded the mean number of chiasmata (41.48) reported for female oocytes (Jagiello et al. 1974). This chiasmata count is actually a gross underestimate of the number of crossovers. More recent molecular maps suggest the number of crossovers in females is likely to be as high as 88 crossovers per nucleus (Broman et al. 1998; Kong et al. 2002). The small sample size in these early studies might contribute to the discrepancies, since later studies suggest considerable variation in number of crossovers between nuclei in humans (Lenzi et al. 2005).

However, the results of another study suggest an alternate explanation—the difficulty of detecting nodules and bars in sectioned nuclei. In electron micrographs of human spermatocytes prepared by microspreading (a method that preserves an entire nucleus in a 2D preparation), Solari (1980) reported an average of 46.2 bars per pachytene nucleus, a number much closer to the 50.6 chiasmata reported by Hultén (1974). In contrast to the Rasmussen and Holm study, Solari found bars in spermatocytes from late zygonema (fully synapsed bivalents) throughout pachynema. (Pachynema in human spermatocytes lasts 16 days.) As is the case of RNs in other species, bars were absent from the pericentromeric regions. Solari (1980) also noted a high frequency of bars near the ends of the bivalents, where Hultén (1974) had reported a high frequency of chiasmata. Although the number of bars were more consistent with the chiasmata counts, Solari (1980) reported a "clustering" of bars on some bivalents and such clusters are unexpected if there is crossover interference. He noted that only one of the bars in each cluster had the usual thickness, implying a difference in morphology of the structures he called bars. Since he made virtually no comment on nodules (on either asynapsed or synapsed axes) in either zygotene or pachytene nuclei, it seems probable that some of the "clusters" structures he classified as bars were in fact nodules. If this were the case, and if only bars are

truly RNs, then the number of crossovers (as measured by “bars”) would be less than 46.2.

There is no doubt that the preparative technique (spreads vs. sections) can dramatically affect the number of RNs observed. For example, in a comparative study in tomato, Stack and Anderson (1986) used a hypotonic bursting technique to make 2D spreads of mid-late pachytene microsporocytes and found an average of 20.5 RNs, a number very similar to the average of 21.3 crossovers per diploid set derived from the linkage map of tomato. They also noted RNs at chiasmata sites at diplonema, confirming the correlation between RNs and crossovers. When they counted RNs in serial sections they found only half as many as they observed in spreads, independent of the stage of pachynema. They suggested that the explanation for the difference lies in the difficulty of detecting RNs in sectioned material.

2.4

Comparisons Between Species

If it is harder to detect RNs in sections, then why do the numbers for some species appear to be more accurate than for others? Besides the difficulties of 3D reconstruction from sections, the fixation, dehydration, embedding and staining methods vary for the different species studied. However, there may also be biological differences between species. For example, *Drosophila* nodules are quite large (100 nm in all dimensions) and extremely electron-dense; both characteristics that facilitate detection. In contrast, plant nodules are less dense and although they measure 100 nm in length relative to the SC, they are only 40–50 nm in width and depth (Stack and Anderson 1986). For a discussion of synapsis and protein foci in *Arabidopsis* see G. Jones and C. Franklin (in this BOOK).

In addition to the RN studies, there are even more significant differences in SyNs in different taxa. SyNs are first observed in zygonema in *Sordaria* and plants, but not until pachynema in *Drosophila*, an organism without a classic zygonema. ANs/SyNs are sometimes apparent on asynapsed axes and at pairing forks in humans, plants, and *Sordaria*, but only on fully formed SCs in *Drosophila*. They also differ in size, shape, and electron density between species. Most notably there are species-specific differences in the ratio of SyNs to RNs as clearly illustrated by comparing the 16 : 1 ratio of SyNs to RNs in plants (Stack and Anderson 1986) to the estimated 1.5–2 ratio in *Drosophila* (Carpenter 1979b). Similar low ratios were also reported for a number of species of animals and fungi. For review see von Wettstein et al. (1984), although these ratios have been revised for animals (see Molecular Components, below).

3

Proposed Models of Synapsis and Recombination

Before moving on to the molecular components of ANs, SyNs and RNs, it may be instructive to consider the two most popular models of molecular mechanisms of synapsis and recombination that were in vogue when RNs and SyNs were first described: the delayed replication model and the double-strand break (DSB) model.

3.1

The Delayed Replication Model

Before the advent of modern molecular techniques, large quantities of meiotic cells were needed for biochemical analysis. *Lilium* was selected for meiotic study because all the microsporocytes in the (large) buds were at the same stage of meiosis and sufficient quantities of buds could be obtained for biochemical studies from commercial fields. Hotta and Stern (1971) found that a sub-fraction (0.1–0.3%) of the genome of lily microsporocytes was delayed in replication until zygonema and that if this replication was blocked homologs did not synapse and the chromosomes fragmented (Hotta and Stern 1971). Stern and Hotta (1974) proposed that this “zygotene DNA” was used in the check for homology that precedes or accompanies synapsis. These observations lead to the delayed replication model.

An often overlooked aspect of this model involves the choice of repair partner. Repair via homologous recombination in mitotic cells requires sister chromatids, while homologous recombination in meiotic cells preferentially utilizes the homolog. This choice of homolog over sister is often credited to meiotic-specific proteins such as DMCI and MSH4 and MSH5 and SC proteins. However, the ultimate means of “selecting” a homologous sequence on a homolog vs. a sister is to force this choice before the DNA is replicated: i.e., before there *is* a sister. Barring the presence of repetitive DNA, this assures choice of homologous sequences on homologous chromosomes independent of the evolution of meiotic-specific proteins.

3.2

The Double-Strand Break Model

Szostack et al. (1983) proposed that the initiating event in meiotic prophase is a double-strand break (DSB). In this model, enzymatic resection of the 5' end produces a single-stranded 3' tail, which is then utilized in a search for homology, followed by invasion of the ssDNA into the homologous duplex. Subsequent processing of the resulting heteroduplex produces a double Holliday junction. Depending on the direction of resolution, this structure can lead to either reciprocal recombination or a gene-conversion event. In

this model, gene conversion is the mechanism by which homology is checked and all molecular events prior to the resolution step are common to both pathways.

Recent studies do not support the original version of this model. Mounting evidence suggests that molecular events associated with synapsis involve a different pathway from that required for crossing over (Borde et al. 2004). However, it is still assumed that the initiating event for both synapsis and recombination is a DSB, followed by resection of the 5' end. Current models propose that homology is checked by a mechanism similar to synthesis-dependent strand annealing (SDSA) (Haber 1999) (J. Haber, in this SERIES), but that only crossovers require a double Holliday junction.

Within the context of these two models, it is relevant to consider the following questions as we examine the location of various meiotic proteins below: (1) When do DSBs occur? (2) What might the presence of Rad51 foci mean, and might its presence mean different things in different organisms? (3) What is the role of MSH4 and MSH5 and do their roles differ in different organisms? (4) What might the location of the various damage checkpoint proteins be able to tell us?

In somatic cells, induction of DSBs rapidly leads to the phosphorylation of the histone variant H2AX (Rogakou et al. 1999). Consequently, an antibody that detects γ H2AX, the phosphorylated form of the histone variant H2AX, is often utilized as an indicator of DSBs. However, there seems to be no definitive experiment demonstrating that phosphorylation of H2AX occurs *only* when there are DSBs, not when there are single-stranded gaps such as those created by stalled replication forks. In mouse spermatocytes, γ H2AX labeling was noted during leptotema of meiotic prophase as a diffuse staining over the entire nucleus and presented as evidence that DSBs occur at this stage (Mahadevaiah et al. 2001). However, the same diffuse γ H2AX nuclear staining has been reported in Intermediate and B spermatogonia—two somatic cell divisions before the spermatogonia differentiate into spermatocytes (become meiotic), long before DSBs are postulated to occur (Hamer et al. 2003). Mahadevaiah et al. (2001) found that during zygonema the general chromatin staining disappeared over the synapsed autosomes, but remained over asynapsed AEs. Prominent γ H2AX staining also remained over the sex body (the chromosomal domain of the asynapsed X and Y chromosomes) and became more heavily stained as pachynema progressed—a week after the spermatocytes had progressed beyond leptotema. This γ H2AX staining of the sex body and persistent staining over asynapsed axes has been attributed to silencing of asynapsed chromosomes (Turner et al. 2005). An explanation for the spermatogonial staining has never been offered. Consequently, interpretation of γ H2AX staining as evidence of DSBs, at least for meiotic cells, should be approached with caution. In summary, the presence of γ H2AX staining is not conclusive proof of DSBs or of the DSB model.

4

Molecular Components

Although relatively few molecular components of ANs, SyNs, and RNs have been identified, it is already apparent that there are considerable species-specific differences in the composition of these complexes. Until recently, molecular components of nodules could only be inferred based on whether a mutation resulted in aberrant or absent nodules. However, as evidenced by studies in *Drosophila*, some recombination defective mutants (*mei-218* and *mei-41*) altered the number and morphology of nodules, while others (*mei-9*) did not (Carpenter 1979a).

More recently, immunohistochemical techniques have become available that allow detection of components of the various types of nodules. Antibodies can be raised against specific proteins and fluorescent or immunogold labels attached either directly to the primary antibody or to secondary antibodies. This technology has revolutionized meiotic studies and many proteins have been identified as components of ANs, SyNs, and RNs. Since it is impossible to include identification of nodule components in the entire array of model organisms, this section will focus on proteins that have been identified in mammals—primarily mice and humans. A summary of the localization patterns is shown in Fig. 1. Other organisms will be discussed only if data from a different species offers a unique insight. The reader is re-

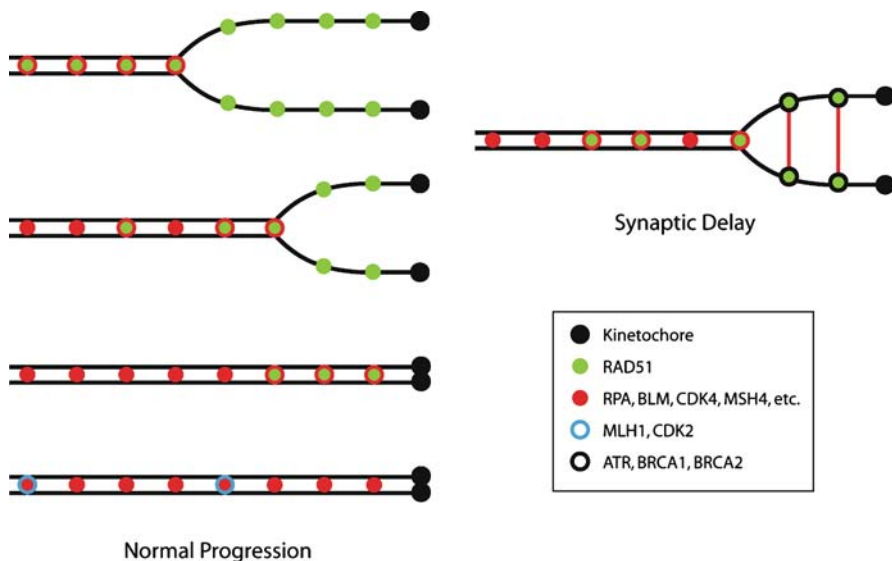


Fig. 1 The localization patterns of AN, SyN, and RN proteins. The diagrams to the left show the distribution of proteins during normal progression of synapsis, while the diagram to the right shows the pattern when there is synaptic delay

ferred to a series of reviews: plants (Anderson and Stack 2005) (G. Jones and C. Franklin, in this BOOK); mammals (Ashley and Plug 1998; Moens et al. 2002). There is also a rapidly growing literature on the meiotic effects of disruption of genes involved in meiosis. For an excellent recent review, see Cohen and Pollard (2006).

The identification of protein components of ANs, SyNs, and RNs, especially those proteins that are known to bind directly to DNA, emphasize the relationship between meiotic chromatin organization and interhomolog interaction. These proteins/protein structures are located at the base of meiotic chromatin loops and are directly associated with the chromatin cores. While these cores are associated with the AEs, there is a distinction between “cores” and AEs. For a full discussion, see J.A. Suja and J.S. Rufas (in this BOOK)¹.

4.1

Components and Potential Components of Axial Nodules

The existence of ANs in multicellular organisms suggests there is assembly of proteins at sites prior to synapsis. The DSB model suggests that DSBs occur early in meiotic prophase while the Delayed Replication Model implies that DSBs are delayed until homology has been verified. Therefore, AN protein complexes fall into two subcategories: (1) proteins for which there is evidence of assembly of a complex before the occurrence of double-strand breaks (DSBs) and (2) proteins that could be assembled either before or after DSBs. Key questions include “When and under what circumstances do ANs assemble? How different are the circumstances of assembly in different species? Are there major differences in the proteins in different species and what do these differences tell us about species variations in meiosis?”

4.1.1

Rad51/DMC1

Rad51 and DMC1, eukaryotic homologs to the bacterial RecA protein, localize at discrete sites along the asynapsed AEs in mammals. The most studied and best understood activity of Rad51 and DMC1 is their ability to polymerize on the 3' resected end of a DSB, then to participate in a homology search, strand invasion and heteroduplex formation. Therefore the presence of Rad51 and/or DMC1 foci is almost universally taken as evidence of the occurrence of a DSB. However, there are additional circumstances under which a Rad51 filament can assemble. Both the bacterial protein RecA (Lusetti and Cox 2002) and the eukaryotic protein Rad51 (Aguilera 2001) bind to ssDNA

¹ Later on, when individual “chromatid cores” separate during diplonema/diakinesis, these core structures are continuous across the chiasmata. Hence, the “bars” mentioned above (Sect. 2.3) may represent transition stages from recombination nodules to chiasmata, where non-sister cores are reconnected at a crossover site.

at delayed replication forks in the single-strand gap between the replicated and unreplicated sequences (see also C. Rudolph et al., in this SERIES). Under these circumstances, RecA can participate in fork regression that permits the restart of DNA replication without generating a DSB (Robu et al. 2001). Therefore an alternate interpretation of the Rad51/DMC1 foci on asynapsed AEs is the accumulation of these proteins on ssDNA at stalled replication forks between the already-replicated bulk DNA and the as yet unreplicated “zygotene DNA”, on which the delayed replication model is predicated. This multiuse property of Rad51 is indicative of the problem of differentiating between replication and recombination models.

Rad51 is essential in mammals as evidenced by the fact that *rad51*^{-/-} mice die early in utero in the absence of external induction of DSBs (Lim and Hasty 1996; Tsuzuki et al. 1996). This is not the case in *S. cerevisiae*. The difference in the necessity for Rad51 may lie in the fact that replication in mammals is discontinuous, requiring restart of stalled forks (Holmquist and Ashley 2006). DMC1 is a meiotic-specific gene required for meiotic progression in both yeast (Bishop 1994; Bishop et al. 1992) and mammals (Pittman et al. 1998; Yoshida et al. 1998).

In mammals and birds, Rad51/DMC1 foci (the original antibody did not differentiate between the two proteins) first appear during leptoneuma and early zygonema, often before formation of AEs (Ashley et al. 1995). (Since synapsis commences shortly after AEs begin to form in mouse spermatocytes, there is no clear separation between leptoneuma and early zygonema.) The number of foci in these early meiotic prophase stages varied between 230–270 in spermatocytes to 350 in oocytes (Moens et al. 1997; Plug et al. 1996). As AEs form, Rad51/DMC1 foci localize along them (Fig. 2). Therefore Rad51 and DMC1 unambiguously qualify as AN components in mammals. There are over 250 Rad51 foci in leptoneuma/zygonema of mouse spermatocytes (Moens et al. 1997; Plug et al. 1996) with many of these foci persisting into early pachynema—nearly 3 days (Van der Meer et al. 1993). The continued presence of so many Rad51 signals during this period argues against a rapid turnover of Rad51/DMC1 in mouse spermatocytes. In mammalian somatic cells, unrepaired DSBs are shunted into an apoptotic pathway within hours. Given that mouse DNA contains nearly 50% repetitive sequences, it seems inconceivable that 250 DSBs could persist for nearly 3 days without devastating consequences for the organism. Under the delayed replication model, the foci do not represent DSBs which would not occur until after homology was verified (following additional unwinding at the forks).

The genomes of vertebrates differ from other organisms in that their DNA is organized into replication clusters whose replication is coordinately regulated [for review, see Holmquist and Ashley (2006)]. In these species, Rad51 might be bound to the ssDNA at the zygotene DNA stalled replication forks. If this is indeed the case, then sequential replication of zygotene DNA sequences

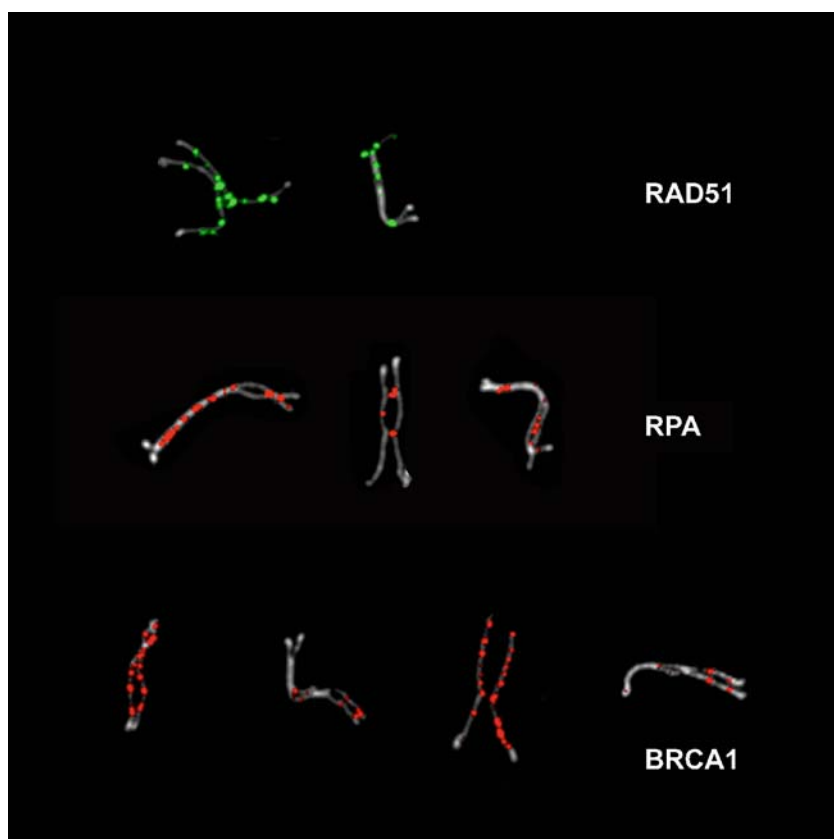


Fig. 2 Distribution of Rad51, RPA, and BRCA1 on AEs and SCs of mouse spermatocytes

within a cluster might explain why mammalian prophase takes so long and why the Rad51 signals persist for nearly 3 days.

The localization of Rad51 foci along the forming AE is likely to be highly significant. Formation of the AE itself appears to occur along the axial core between sister chromatids (J.A. Suja and J.S. Rufas, in this BOOK). The Rad51 bound sequences lie at the base of the chromatin loops associated with the core. This organization excludes any chromatin in the loops themselves from the “search for homology” and binding of other chromatin directly to the axial core/axial element proteins may excluded these sequences from direct participation.

In yeast, Bishop (1994) found that Dmc1 and Rad51 foci colocalize in meiosis just prior to synapsis of homologs. The timing of their appearance led Bishop (1994) to propose that subsequent to DSBs both proteins are involved in nucleoprotein filament formation, the search for homology, and strand invasion and were components of early nodules. However, he noted that the

average (45) or even the maximum number (64) of Rad51 foci in *S. cerevisiae* was far fewer than the expected number of crossovers (87) based on the total length of the genetic linkage map of 4350cM [see Bishop (1994)]. Yet Rad51 and Dmc1 are proposed to polymerize on ss-tails of all DSBs – those giving rise to gene conversion events, as well as those leading to crossovers. Based on estimates by Fogel et al. (1988), Bishop calculated the expected total number of DSBs and exchanges as 175–260 per nucleus and concluded that only about one-half to one-third of yeast gene conversion events are associated with reciprocal exchanges (crossovers). Despite the fact that meiosis proceeds very rapidly (5.5–6.5 h) in *S. cerevisiae*, he postulated that asynchrony of Rad51/Dmc1 complex formation (and rapid progression of the Rad51/Dmc1-dependent steps) during early meiotic prophase account for this shortage.

Obviously the ratio of Rad51 foci to crossovers also differs dramatically between yeast and mammals. Although the number of Rad51/Dmc1 foci in yeast is insufficient to account for all of the estimated crossovers, much less the gene conversion events. The number in mammals is more than ten times the number of crossovers. The 10 : 1 ratio of RAD51 foci to crossovers in mammals is more consistent with Rad51/DMC1 being components of ANs and SyNs involved in a check for homology with only a subset eventually becoming involved in crossing over.

As will be discussed below, several laboratories have proposed a concept of a “pre-recombination” or “pre-synaptic” complex. Consistent with this idea in some mammalian spermatocytes Rad51/DMC1 foci near pairing forks appeared to lie at corresponding sites along the axes of the two homologs (Plug et al. 1996). Subsequent studies have found that the number of Rad51/DMC1 foci in zygonema is approximately half the number of RPA (Replication Protein A) noted in late zygonema/early pachynema (Moens et al. 2002; Plug et al. 1998). Since both Rad51 and DMC1 bind to ssDNA these localization studies are consistent with the hypothesis that sequences on each homolog are loaded with Rad51/DMC1 prior to synapsis and that corresponding sites on the two homologs may interact to participate in inter-homolog interaction.

Once mouse homologs synapse, Rad51/DMC1 foci are prominent at sites along the newly synapsed autosomal SCs, but disappeared relatively rapidly from the autosomal SCs as the spermatocytes enter pachynema (Ashley et al. 1995; Barlow et al. 1997; Moens et al. 1997; Plug et al. 1996). However, if there is a delay in synapsis, such as occurs when a chromosome aberration results in translocation of a portion of one chromosome to another, the Rad51 foci remain on asynapsed AEs. Since the sex chromosomes have no homologs and therefore never completely synapse, it is not surprising that Rad51/DMC1 foci persist on the asynapsed X chromosome axis (Ashley et al. 1995). Although Bishop (1994) and Ashley et al. (1995) proposed for yeast and mice, respectively, that Rad51/DMC1 were likely components of early nodules. However, it was electron microscopic immunogold localization of Rad51/DMC1 antibodies to early nodules in lily microsporocytes that provided the conclusive proof

that these proteins are indeed components of early nodules (SyNs) in plants (Anderson et al. 1997) and mice (Moens et al. 1997). Since SyNs have never been identified in yeast, a similar conclusion for this species is premature.

4.1.2

Other Mammalian Axial Nodule Proteins

Several other mammalian proteins localize at sites along asynapsed AEs and therefore qualify as potential components of ANs. These include ATR (Baart et al. 2000; Keegan et al. 1996; Moens et al. 1999), BRCA1 (Scully et al. 1997b), BRCA2 (Chen et al. 1998), and TopBP1 (Perera et al. 2004). Each of these proteins has been identified as components of the damage control network of cell cycle proteins, which directly or in collaboration with other factors, sense DNA damage or the presence of stalled replication forks and initiate a signaling cascade. Several members of this constellation of proteins also recruit the machinery for repair of the DNA damage and even play a role in determining *which* repair pathway is utilized. DNA repair falls into two major categories: homologous recombination and nonhomologous end joining. Homologous recombination requires more sequence homology, but is precise, with no loss or alteration in information content. Nonhomologous end joining requires less homology and results in loss of a few base pairs at the site of the break. Not only does meiosis require high-fidelity repair in order to assure transmission of all the genetic information, reciprocal recombination requires that homologous recombination occur between homologous chromosomes rather than sister chromatids.

The first of these additional AN proteins to be identified was ATR (Keegan et al. 1996), a phosphoinositide-3-kinase related kinase closely related to ATM. ATM is the gene mutated in the human autosomal recessive disorder ataxia telangiectasia (Lavin 2004; Shechter et al. 2004a; Shiloh 2001). Both ATR and ATM are central components of the DNA damage network of cell cycle checkpoint proteins. Their pathways are nonlinear with many feedback loops, several of which interconnect: see recent reviews by McGowan and Russell (2004); Shechter et al. (2004a). Several other proteins in these pathways are also components of ANs. While ATM is required for the early response to ionizing irradiation and other agents that induce DSBs, the primary response of ATR is to a wide range of agents that cause replication forks to stall in mammalian somatic cells [for review, see McGowan and Russell (2004); Shechter et al. (2004a)]. Although the primary activity of ATR appears to be associated with stalled replication forks, it can bind to the 3' resected end of DSBs independent of the replicative state of the DNA [for discussion, see Shechter et al. (2004b)]. ATR is constitutively associated with another protein ATRIP (Ball et al. 2005; Zou and Elledge 2003). Together they bind to ssDNA on which RPA has polymerized. RPA is a three-subunit protein and the primary eukaryotic ssDNA binding protein required for DNA replication, recombination and re-

pair [for review, see (Wold 1997)]. For further discussion of ATR relative to the two models of early meiotic events see the subsection on the "Potential roles of axial nodule proteins in mammalian meiotic checkpoint control".

Since fluorescent antibodies to ATR colocalize with differentially labeled antibodies to Rad51 on asynapsed AEs (Baart et al. 2000; Keegan et al. 1996), by inference it can be considered a component of ANs. However, Moens et al. (1999) detected Rad51, but not ATR in ANs of mouse spermatocytes by immunogold labeling. This might be a technical failure, since ATR binds to ssDNA, as does Rad51 and if synapsis is progressing normally, there may be little ATR on the AEs. Moreover, although RPA is the substrate for ATR binding, there was little detectable RPA on the AEs when the first small ATR foci appear on the AEs. (For a discussion of RPA on SCs of autosomes, see the next section.) However, if homologs remained asynapsed, both RPA (discussed below) and ATR accumulated on the AEs (Baart et al. 2000; Keegan et al. 1996; Moens et al. 1999). In normal spermatocytes this accumulation is most evident on the X chromosome axis. Delay in autosomal synapsis also leads to accumulation of ATR on the asynapsed AEs; the longer the delay, the greater the accumulation of RPA and ATR. However, while the RPA signal remains focal in nature, the ATR antibody eventually appears to coat the asynapsed axes. When mouse spermatocytes first enter pachynema there are very few RPA foci on the X chromosome axis (Ashley et al. 2004), but as the spermatocytes progress through this long stage (7 days), the number of RPA foci increases, then decreases and disappears by mid-pachynema (about 3 days into pachynema). Yet heavy ATR staining on the X-axis and over sex body chromatin (chromosomal domain of the X and Y) persists into diplonema. Given the widely held assumption that RPA is the only substrate for ATR binding, it is curious that ATR continues to accumulate in the apparent absence of RPA. Turner et al. (2004, 2005) have linked this persistent ATR staining to silencing of transcription of the sex chromosomes in mammalian spermatocytes, but have offered no explanation for the discrepancy between the presence of ATR and the lack of RPA.

Brca1 and *Brca2* are genes that confer a predisposed to breast, ovarian, and a variety of other cancers in those individuals carrying a germline mutation (Scully and Livingston 2000; Welcsh and King 2001). Although the genes are structurally unrelated, their functions overlap [for reviews see Deng (2006) and Zhang and Powell (2005)]. At the cellular level, deficiency of either gene leads to genome instability. In culture, both *Brca1* and *Brca2* deficient cells accumulate spontaneous chromosome aberrations including chromosome and chromatid breaks and triradial and quadriradial chromosome configurations indicative of defective mitotic recombination. Both genes' products are found in a complex with Rad51 in somatic cells and both are implicated in the channeling of repair of DSBs into the homologous recombination pathway rather than nonhomologous end joining [for reviews, see Venkitaraman (2001); Zhang and Powell (2005)]. BRCA1 is a mediator in the damage control net-

work and can be phosphorylated by either ATR (Scully et al. 1997a) or ATM (Cortez et al. 1999; Gatei et al. 2000). There is currently no evidence of phosphorylation of BRCA2 by either ATR or ATM (Venkitaraman 2001).

Like ATR, BRCA1 (Scully et al. 1997b) and BRCA2 (Chen et al. 1998) colocalize with Rad51 on the asynapsed AEs of human spermatocytes and, by inference, are components of ANs. BRCA2 binds Rad51 directly (Wong et al. 1997). BRCA1 and BRCA2 not only colocalize in mitotic and meiotic cells (Chen et al. 1998), they physically associate with one another, although the interaction may not be direct, but through a complex that includes several other proteins including Rad51 [see discussions in Dong et al. (2003); Venkitaraman (2001)]. The cytological profiles of the BRCA1 and BRCA2 foci in human spermatocytes follow the general pattern of ATR observed in mouse spermatocytes. The size of both the BRCA1 and BRCA2 foci is minimum during normal zygonema and rapidly disappear once homologs synapse. However, if there is a delay in synapsis, the foci increase in size. As is the case with Rad51 foci, BRCA1 foci on homologous AEs often appear to lie at corresponding sites along the AEs (Fig. 2). If asynapsis persists, then the protein (detected by the appropriate antibody) eventually appears to coat any asynapsed AE, including that of the X chromosome [BRCA1: (Scully et al. 1997b); BRCA2: (Chen et al. 1998)]. However, there is no accumulation of these proteins on the chromatin within the sex body. For discussion of BRCA1 and BRCA2 relative to the two models of early meiotic events see the subsection on the "Potential roles of Axial Nodule proteins in mammalian meiotic checkpoint control".

TopBP1 (topoisomerase II β -binding protein 1), yet another checkpoint protein, was initially identified through its association with topoisomerase II β (Yamane et al. 2003). It has a BRCT domain that allows it to interact with BRCA1. In mammalian somatic cells TopBP1 localizes to sites of DNA damage in response to both DSBs and stalled replication forks (Honda et al. 2002; Yamane et al. 2003). During meiosis TopBP1 localized to assembling AEs as early as leptoneuma/early zygonema. Interestingly, it exhibits only 10–20% colocalization with DMC1 foci during this time, but almost perfect colocalization with ATR (Perera et al. 2004). TopBP1 remains associated with AEs into pachynema when the only remaining asynapsed AEs in normal spermatocytes are those of the X and Y (Perera et al. 2004). By mid-pachynema TopBP1 staining, like that of ATR, includes the sex body chromatin.

4.1.3

Spo11 and the Mre11/Rad50/Nbs1 (MRN) Complex

For this discussion it is helpful to begin with the evidence from yeast (see also S. Keeney, in this BOOK). Although Spo11 and Mre11/Rad50/Xrs2 (MRX)²

² Mre11/Rad50/Xrs2 or MRX is the yeast equivalent of the mammalian Mre11/Rad50/Nbs1 or MRN complex (see also K.-P. Hopfner, in this SERIES).

are prime candidates for components of ANs and/or SyNs, there is no conclusive cytological evidence to substantiate this supposition in fungi. Nonetheless genetic evidence from yeast supports the existence of a complex of ten proteins required for the initiation of recombination in *S. cerevisiae* (Borde et al. 2004): Mre11p, Rad50p, Xrs2p, Spo11p, Rec102p, Rec103, Rec104p, Rec114p, Mer2p, Mei4p³. All ten proteins are necessary for formation of DSBs. No homologs or orthologs of these "Rec" proteins have been identified in other organisms. They have no domains that offer clues to their function. Mer2p and Mei4p are splicing factors, and their inclusion in this complex also remains elusive. However, the remaining proteins have known functions. Spo11p is the phylogenetically conserved, meiotic-specific, topoisomerase responsible for making programmed DSBs during meiotic prophase [yeast: (Keeney et al. 1997); mammals: (Keeney et al. 1999; Metzler-Guillermain and de Massy 2000; Romanienko and Camerini-Otero 1999); plants: (Hartung and Puchta 2000)]. However, Spo11p is also required for binding of the other proteins in the complex and a mutation in any one of these proteins blocks the formation of DSBs (Borde et al. 2004), suggesting that protein composition and conformation of the complex itself is critical for break formation. Furthermore a mutation in Spo11 in *S. cerevisiae* results in a 25% truncation of premeiotic S phase (Cha et al. 2000) suggesting Spo11p may play a critical role in slowing or delaying some aspect of meiotic DNA replication. Yeast Spo11 is also required for homologous pairing (Loidl et al. 1994; Weiner and Kleckner 1994) and both AE and SC formation (Dresser and Giroux 1988; Giroux et al. 1988). Although the *S. cerevisiae* data suggest that DSBs occur about the time the SC is forming, the deficiency in number of Rad51 foci suggests synaptic association sites in this species may be involved in crossovers, but not necessarily gene conversion. Might the DSBs only be necessary for crossovers, with a check for homology occurring prior to these breaks?

In contrast, the *Drosophila spo11* homolog (*mei-W68*) is not required for homologous pairing and SC formation in *Drosophila* (McKim et al. 1998; McKim and Hayashi-Hagihara 1998) despite the fact that RNs were morphologically defective and recombination virtually eliminated in two *mei-W68* mutants (Carpenter 2003). Taken together these results strongly imply that DSBs occur after synapsis in *Drosophila*. A similar type of mutation analysis in *Caenorhabditis elegans* suggests that synapsis and SC formation also precede DSB formation in worms (Dernburg et al. 1998).

Although Spo11 is required for SC formation in mammals, AE formation appears normal in its absence (Baudat et al. 2000; Romanienko and Camerini-Otero 2000). No information is yet available on Spo11 has any effect on premeiotic DNA synthesis in this group of organisms. An antibody raised against Spo11 produced small foci (a diffuse pattern) at numerous sites in leptotene spermatocyte nuclei of the mouse, although most of these did

³ Yeast nomenclature adds a "p" to the gene designation for the protein.

not localize along the axial elements, but throughout the nucleoplasm (Romanienko and Camerini-Otero 2000). As homologs synapsed the antibody did not produce discrete foci. Instead, it coated the entire SCs. Based on this (sole) study, there is no support for the supposition that Spo11 is a component of ANs or SyNs in mammals.

Homologs of *mre11* and *rad50* have been identified in all eukaryotes examined to date. While Xrs2 is not conserved in mammals, it is replaced by Nbs1 (Varon et al. 1998). The yeast MRX complex and the mammalian MRN complex assemble at sites of DSBs in mitotically dividing cells and play an important role in DNA repair. For review, see Assenmacher and Hopfner (2004); Stracker et al. (2004) and K.-H. Hopfner (in this SERIES). In addition, the mammalian MRN complex is often pre-positioned at sites vulnerable to breaks such as the hairpin structure at the fold-back of palindromic sequences (Paull and Gellert 1999) or at replication origins (Maser et al. 2001). Consistent with meiotic prepositioning, the yeast ten protein "pre-recombination complex" binds to DNA sequences near or at DSBs sites *after* the sequences have replicated, but *before* breaks occur (Borde et al. 2004). This is not the case in *Arabidopsis* where the presence of Rad50 and Mre11 is not required for DSB formation (Puizina et al. 2004) but for DSB processing and strand resection (G. Jones and C. Franklin, in this SERIES).

Despite the fact that Mre11 and Rad50 relocate from a diffuse nuclear distribution to discrete foci in response to DSBs in mammalian somatic cells [see Lisby et al. (2004); Maser et al. (1997); Mirozoeva and Petrini (2003); Nelms et al. (1998)], immunolocalization of members of the Rad50/Mre11/Nbs1 complex to ANs in meiotic cells has proven elusive in mammals (Eijpe et al. 2000). However, Eijpe et al. concluded that Mre11 and Rad50 were part of a "pre-recombination complex" involved in chromatin restructuring prior to synapsis and recombination. (Note that the use of this term in mammals pre-dates that in yeast and does not necessarily mean the same thing.) Their conclusion was based on the fact that both the Rad50 and Mre11 antibodies produced a diffuse general chromatin staining in pre-leptotene fixed and sectioned testis of mouse and rat spermatocytes, a pattern that persisted until homologs synapsed. [Results of fluorescent antibody localization (FAL) attempts on microspread preparations from mammalian material were inconclusive.] The staining reaction in sections disappeared over the chromatin as soon as the autosomes synapsed, but persisted over the sex body (the chromosomal domain of the X and Y) until diplonema (Eijpe et al. 2000). They never observed reproducible discrete foci over the asynapsed AEs, the pattern predicted for a component of ANs.

Enzymatic functions of the MRX (yeast) or MRN (mammals) complex include dsDNA-dependent 3'-5' exonuclease activity, ssDNA-dependent endonuclease activity and weak helicase (unwinding) activity (Connelly and Leach 2002; D'Amours and Jackson 2002). Although the exonuclease activity of the complex generates a 5' ssDNA tail, instead of the 3' tail that is actually

observed in meiosis, the yeast MRX complex does appear to be required for a step immediately following DSB formation. In separation of function mutants of yeast, the MRX complex is required for removal of Spo11p from the 5' ends of the DSBs and subsequent resection (Haber 1998; Nairz and Klein 1997). Therefore, despite the fact that its exact function in DNA repair in yeast is not yet clear, the MRX complex seems to play an important role in meiotic progression following the formation of DSBs. In addition to its direct involvement in DNA repair, the MRX/MRN complex is also a "break sensor" and intimately involved in activation and propagation of cell cycle checkpoint signaling in response to DNA damage in both somatic and meiotic cells. For a recent review of the mammalian MRN complex and a summary of the differences in the meiotic behavior of mutations in *Mre11*, *Rad50* and *Nbs1* in mammals versus yeast see (Stacker et al. 2004) (see also K.-H. Hopfner, in this SERIES).

In summary, although there is ample reason to presume that there is a protein complex that includes Spo11, Mre11, Rad50, and Nbs1/Xrs2 that binds to chromatin sites prior to DSB formation, antibodies applied to spread or sectioned material in mammals have not localized as discrete foci on AEs along SCs. Although current evidence does not support the supposition that Mre11, Rad50, and Nbs1 are components of ANs in mammals, the possibility has not been ruled out. As discussed below, some proven components of ANs and SyNs are known to interact with these proteins.

4.2

Potential Roles of Axial Nodule Proteins in Mammalian Meiotic Checkpoint Control

Proteins involved in detection of stalled replication forks and early signaling of delayed replication differ from those involved in detection and initial signaling of damage incurred from DSBs. Key proteins in the "delayed replication" network include ATR, BRCA1 and BRCA2, all components of ANs that localize on asynapsed AEs and accumulate when there is a delay in synapsis. However, each of these proteins can eventually be called into play if there is a DSB, Rad51 and RPA can polymerize on the resected 3' ssDNA tail. RPA can then provide a substrate for binding of ATR, ATR interacting protein (ATRIP) and TopBP1 (Jazayeri et al. 2002). BRCA1 and BRCA2 both bind Rad51 and BRCA1 can be phosphorylated by either ATR or ATM [for review see Venkitaraman (2001)]. Therefore the localization pattern of these proteins in mammals does not currently provide a means of discriminating between the two models. *S. cerevisiae* has *Atr/Atm* homologs. *MEC1* is more closely related to *Atr*, while *TEL1* is more closely related to *Atm* (Craven et al. 2002). Although there appears to be even more overlap in function between the two orthologs, the primary function of *Mec1* does appear to be in DNA replication. Besides mediating S-phase

checkpoint responses, it prevents replication fork collapse in replication slow zones (Cha et al. 2000). During meiosis *MEC1* is required for recombination (Grushcow et al. 1999).

Meiosis in mammals is a prolonged affair. In mouse spermatocytes, meiosis occurs over a 2-week period (Oakberg 1956). In female eutherian (placental) mammals, the meiotic prophase occurs prenatally. The oocytes then arrest until puberty, which in humans is more than a decade later. Two checkpoints have been identified in males: one temporally located in mid-pachynema, the other at metaphase I. The mid-pachytene checkpoint is so named because that is the time the spermatocytes become apoptotic as judged from the histology of the testis sections (Ashley et al. 2004; de Rooij and de Boer 2003). Yet based on cytological evaluation, the spermatocytes appear to have arrested in zygonema, since almost all mutations that arrest at this checkpoint are asynaptic (Ashley 2002; de Rooij and de Boer 2003). Therefore, the presence of so many key cell cycle proteins on asynapsed axes is of obvious interest. In females there is a high attrition rate in prophase oocytes even in normal animals. However, some mouse oocytes homozygous for a number of mutations in mouse male spermatocyte progress through prophase (Cohen and Pollard 2006). In addition, the metaphase I checkpoint appears to be less stringent in females than in males (LeMaire-Adkins et al. 1997).

Genetic evidence from mice supports a role for BRCA2 in synapsis, at least in males. Despite the fact that *Brca2* mutations are embryonic lethals when homozygous in mice, a BAC transgene that expresses the human *Brca2* gene in somatic tissue, but not in the germline, allowed an evaluation of the meiotic phenotype. Spermatocytes exhibited synaptic defects and a reduced number of Rad51 foci (Sharan et al. 2004), although synapsis in oocytes appeared normal.

Although BRCA1 foci are rapidly lost as homologous chromosomes synapse in normal animals, the only mouse *Brca1* meiotic construct did not reveal a synaptic phenotype (Xu et al. 2003). However, the study did find that BRCA1 activity is essential for reciprocal recombination. The requirement for BRCA1 in this step is not surprising since one of its roles in the damage checkpoint pathway is to shunt repair into the homologous recombination pathway instead of the nonhomologous end joining. In this regard it is curious that BRCA1 localizing on AE since homologous recombination before synapsis would be between sister chromatids instead of homologs.

Further complicating an understanding of the interaction of these DNA damage checkpoint proteins in meiosis, BRCA1 has been shown to colocalize and coprecipitate with Rad50 as well as Mre11 and Nbs1 (Wang et al. 2000; Zhong et al. 1999) and ATR can phosphorylate Mre11, providing yet another interconnection in this snare of repair and checkpoint control proteins (Costanzo et al. 2001). Although the mechanisms of the potential interactions and the circumstances under which they occur are unclear, it is noteworthy that all of these cell cycle proteins are potentially in the same complex (ANs)

in early meiotic prophase. It is also important to keep in mind that other taxa do not always have clear orthologs of several of these proteins. This lack may well explain some differences in meiotic progression between organisms.

4.3

Synaptic Nodules

This category of proteins includes all those that are detected at multiple sites along the SCs in zygonema and early pachynema. Many of these are involved in DNA replication and/or repair. Consistent with the EM observations of the apparent assembly of SyNs at pairing forks many of these proteins first become evident at forks. In contrast to AN proteins that accumulate if there is asynapsis, but are rapidly lost after SC formation, the SyN protein antibodies increase in intensity as homologs synapse. The foci then gradually decrease in intensity, size, and number during the early- to mid-pachynema transition. Although synapsis is generally considered complete as soon as the SC is formed, the continued presence of multiple SyN proteins along these SCs suggests that the molecular processes associated with synapsis are not completed until well into pachynema [for discussion, see Ashley et al. (2004)].

RPA, a heterotrimeric eukaryotic protein complex that binds to ssDNA is essential for DNA replication, repair and recombination. Since it has no other known substrate, cytological detection of RPA can be considered an indicator of the presence of ssDNA. Although a few small RPA foci are visible on asynapsed AEs (de Vries et al. 2005; Moens et al. 2002), most RPA foci are found along the SCs during zygonema and early pachynema (Fig. 2), first becoming evident at pairing forks where they colocalize with Rad51 foci (Moens et al. 2002; Plug et al. 1997b, 1998). The maximum number of RPA foci observed in mouse early pachytene spermatocytes is 150–180, approximately half the maximum number of Rad51 foci (Ashley et al. 2004; Moens et al. 2002; Plug et al. 1998). Between early and mid-pachynema all autosomal foci on the SCs disappear (Ashley et al. 2004), suggesting that the DNA sequences involved in the check for homology accompanying synapsis are now restored to a double-stranded state. There is also a prominent RPA focus at the base of the pairing region between the X and Y, the site of an obligatory crossover between the X and Y.

Although small RPA foci are visible on the asynapsed autosomal AEs throughout zygonema, synaptic delay results in much more prominent RPA foci. Many of these span the distance between asynapsed AEs appearing “bar-like” (Fig. 2). If the size and intensity of the RPA foci reflect the amount of ssDNA, the RPA foci on the delayed AEs suggest more ssDNA at these sites [for discussion, see Plug et al. (1998; 1997b)]. In the case of the X chromosome numerous RPA foci appear and disappear between early and mid-pachynema, suggesting that ssDNA at these sites is also being processed (Ashley et al. 2004).

Mutations in the human *BLM* gene give rise to the rare autosomal recessive disorder Bloom Syndrome (Ellis et al. 1995). Individuals with this disorder

have a growth deficiency, sun-sensitive pigmentary lesions of the skin, and are immunodeficient as well as extremely sensitive to ionizing irradiation and predisposed to a variety of cancers [for review see, German and Ellis (1998)]. Cell cultures from these individuals exhibit genomic instability and chromosome aberrations such as chromatid breaks and triradial and quadriradial chromosome configurations that are thought to arise as a result of replication errors (see German and Ellis (1998)). BLM is a member of the RecQ helicase family of 3'-5' ssDNA helicases that recognize, bind to, and expand single-strand gaps in duplex DNA (Ellis et al. 1995).

Although some general chromatin staining is visible throughout mouse spermatocyte nuclei during leptotema and early zygotema (Moens et al. 2000), an antibody to the BLM protein primarily localizes at sites along the SCs in mouse spermatocytes (Moens et al. 2000; Walpita et al. 1999). BLM foci appear slightly later than RPA, but colocalize with RPA (Moens et al. 2000; Walpita et al. 1999). As is the case with the RPA antibody, the number of BLM foci drops progressively during early pachytene until none are evident by mid-pachytene (Walpita et al. 1999). There is always a prominent BLM focus during early pachytene at the base of the XY in the pseudoautosomal region where an obligatory crossover occurs between the X and Y (Moens et al. 2000; Walpita et al. 1999). Additional BLM foci appear and disappear at multiple sites along the AE of the X chromosome during early to mid-pachytene. There are no reports of the meiotic behavior of BLM foci when there is delayed autosomal synapsis. However, later in pachytene, the BLM signal became more dispersed throughout the entire nucleoplasm, raising the possibility that it may play a later additional role in meiosis (Walpita et al. 1999).

Given the facts that BLM directly interacts with topoisomerase III α (Topo III α) and the two proteins colocalize in somatic cells, coimmunoprecipitate in human cell extracts, and bind to one another in vitro, it is not surprising that they colocalize in human spermatocytes (Johnson et al. 2000). However, Johnson et al. (2000) also reported Topo III α on AEs before synapsis. If confirmed, Topo III α should be considered a component of both ANs and SyNs.

In somatic cells Cdk4, with its various Cyclin D partners, triggers the transition from G1 to S phase (Zhang 1999). Given the temporal role of Cdk4, it was unexpected that antibodies to Cdk4 localize at sites along the newly synapsed SC in mouse prophase spermatocytes (Ashley et al. 2001). The temporal and spatial patterns of localization of Cdk4 on the SCs follow the same profile as RPA and BLM. There is progressive loss of Cdk4 foci from the autosomal SCs during early pachytene and Cdk4 foci appear and disappear from sites along the AE of the X chromosome. There is no general chromatin staining over either the autosomes or the sex body with the Cdk4 antibody.

MSH4 and MSH5 form a heterodimer and are meiotic-specific members of the subfamily of mismatch repair proteins called MutS homologs [see Svetlanov and Cohen (2004)]. Unlike other members of this subfamily, they do not

recognize mismatches in DNA [reviewed by Hoffman and Borts (2004)]. Yet they are indispensable for reciprocal recombination in *Saccharomyces cerevisiae* (Hollingsworth et al. 1995; Ross-Macdonald and Roeder 1994), *C. elegans* (Kelly et al. 2000; Zalevsky et al. 1999), *Mus musculus* (de Vries et al. 1999; Edelmann et al. 1999; Kneitz et al. 2000), and *Arabidopsis thaliana* (Higging et al. 2004). Disruption of either the mouse *Msh4* (Kneitz et al. 2000) or *Msh5* gene results in asynapsis (de Vries et al. 1999; Edelmann et al. 1999).

In mouse spermatocytes (Kneitz et al. 2000; Santucci-Darmanin et al. 2000) and oocytes (Kneitz et al. 2000) MSH4 colocalized with RPA at multiple sites along the newly synapsed SCs and disappeared by mid-pachynema. Unlike RPA, BLM and Cdk4, MSH4 did not localize to sites along the asynapsed X axis (Santucci-Darmanin et al. 2000), suggesting a specific role in inter-homolog interactions, rather than a general role in processing of ssDNA. There is no data on localization of MSH4 when there is delayed synapsis. In human oocytes a few MSH4 foci are present in leptoneuma, but do not appear to be associated with the AEs until zygonema (Lenzi et al. 2005). Lenzi et al. (2005) observed the maximum number of foci associated with the SCs in late zygonema. During pachynema the number of MSH4 foci steadily declined with only a few remaining by late pachynema. Antibodies to MSH5 have only recently become available (Lenzi et al. 2005). In human oocytes, MSH5 colocalizes on SCs with MSH4, as would be predicted for a heterodimer partner. Not surprisingly, the distribution pattern and number of MSH5 foci observed at different stages of prophase mirrors those observed for MSH4 (Lenzi et al. 2005).

Pol β possesses both polymerase and deoxyribose phosphatase activities and is involved in DNA base excision repair (BER) (Idress et al. 2002). Pol β foci occur on both the asynapsed AEs and the newly synapsed SCs in zygonema (Plug et al. 1997a). Plug et al. (1997a) found that some incompletely synapsed zygotene bivalents seemed to have an accumulation of Pol β at pairing forks. Although the number of foci along the autosomal axes decreased during pachynema, comet-shaped streamers of Pol β signal were visible on one or both sides of several bivalents and persisted into diplonema. In addition, by mid- to late- pachynema a Pol β signal became evident at each end of the autosomal bivalents and the AEs of the X and Y chromosomes. Both the "streamer-like" signal and the telomeric localization in late pachynema suggest a role for Pol β in addition to its currently unidentified function in SyNs.

With the exception of Cdk4, the SyN proteins identified so far are all involved in multiple aspects of DNA metabolism including replication, repair, and recombination. Therefore their presence, either singly or in combination with the others, does not provide conclusive evidence of involvement in one of these pathways to the exclusion of any other. Even the roles of MSH4 and MSH5, which seem so clear-cut in yeast, are much less so in other organisms. For example, in *Coprinus cinereis* the Msh5 homolog (formerly called Spo22) is required for premeiotic DNA synthesis (Cummings et al. 2002).

As was the case with the AN proteins, there is evidence of interactions between SyN proteins. For example, different portions of the BLM molecule can bind to topoisomeraseIII α (TopoIII α), Rad51, MLH1, BRCA1, ATM, p53, Fen1, and Mre11/Nbs1 [see Cohen and Pollard (2006)]. In vitro BLM, in cooperation with TopoIII α can effect the resolution of recombination intermediates (double Holliday junctions) resulting in nonreciprocal resolution (Wu and Hickson 2003).

4.4

Potential Relationships Between AN and SyNs

“Early nodules” (ANs and SyNs) on AEs (and SCs) in plants and animals have generally been reported as variable in size. In addition, structures too small to be considered nodules have been observed on the asynapsed AEs, especially in plants. However, synaptic delay of the type that has led to the accumulation of proteins such as ATR, TopBP1, BRCA1, and BRCA2 in mammals might result in accumulation of sufficient proteins for these small structures to be re-classified as “nodules”. The formation of SyNs at synaptic forks must also be reconsidered. Although SyNs appear to form at pairing forks or “convergences”, this appearance may be deceptive. Given the presence of Rad51 in both ANs and SyNs, the possibility that ANs morph into SyNs through assembly of additional proteins required for processing the ssDNA deserves serious consideration. The appearance of new proteins such as RPA, BLM, TopoIII α , MSH4 and MSH5, Cdk4, Pol β , and Chk1 at pairing forks seems to support this view. Rather than forming de novo, nodules may merely “grow” to a recognizable size as they acquire the proteins necessary for molecular events associated with synapsis. If there is a delay in synapsis, they may “grow” through the acquisition of different proteins as they unwind more ssDNA, bind more Rad51, RPA and other proteins such as ATR, BRCA1 and BRCA2.

As mentioned above the number of foci (120-150) of both RPA (Plug et al. 1998) and MSH4 foci (Lenzi et al. 2005) is roughly half the estimated number of Rad51 foci (250–300) observed in leptotene/early zygotene nuclei (Ashley et al. 1995; Moens et al. 1997). Taken together with the observation of several AN proteins at corresponding sites on homologous AEs, these numbers support the concept of “presynaptic” or “prerecombinational” complexes at preselected DNA sites.

4.5

Recombination Nodules

The first component of RNs to be identified was MLH1 (Baker et al. 1996), which belongs to the MutL subfamily of mismatch repair proteins. During meiosis it forms a heterodimer with MLH3, another member of this subfamily [for review, see Kolas and Cohen (2004)]. MutL dimers are recruited

to specific sites by their MutS partners, and in meiotic recombination those MutS partners are MSH4 and MSH5 [see Kolas et al. (2005); Wang et al. (1999)]. The MLH1/MLH3 heterodimer interacts with double Holliday junction intermediates that give rise to crossovers during meiosis (Kirkpatrick 1999).

Baker et al. (1996) found that an antibody to MLH1 localized as foci at a few sites along the SCs in mouse. Evidence that these foci represent sites of crossovers was similar to the evidence originally presented for involvement of RNs in crossover by Carpenter (1975): 1) The number and distribution of observed MLH1 foci corresponded to the number and distribution of reported chiasma, 2) no MLH1 foci were observed in heterochromatin where crossovers are known to be suppressed, 3) there was an MLH1 focus in the pseudoautosomal region between the X and Y chromosomes where an obligatory crossover occurs, and 4) in mouse oocytes MLH1 foci persist into diplotene where they clearly localize at chiasmata sites. A role of MLH1 in reciprocal recombination was confirmed by examination of *Mlh1*^{-/-} mouse spermatocytes (Baker et al. 1996; Edelmann et al. 1996). Male mice deficient for *Mlh1* are viable, but sterile with an arrest at metaphase I (Baker et al. 1996; Eaker et al. 2002; Edelmann et al. 1996). Synapsis in spermatocytes appeared to proceed normally. However, between mid- to late-pachynema first the XY bivalent, then the autosomal bivalents, begin a precocious desynapsis and fall apart, behavior consistent with a lack of a crossover. Interestingly, the chromosomes appear intact, suggesting that DSBs are repaired despite the defect. Oocytes of female *Mlh1*^{-/-} mice proceed through pachynema, but likewise are defective in crossover and chiasmata formation (Woods et al. 1999). Oocytes arrest in dictyate⁴ and the univalents lead to severe metaphase I spindle defects, and also result in sterility. If any doubt remained as to whether or not MLH1 was a component of RNs, immunogold antibody staining has shown that MLH1 localizes to RNs in electron micrographs (Kolas et al. 2005; Moens et al. 2002).

In spermatocytes of the mouse strain C57Bl6 all MLH1 foci form during a relatively short period (about the end of the first day of pachynema, which lasts 7 days) and the number remains constant until 3–4 days later when their number begins to drop (Ashley et al. 2004). As mentioned above, MSH4 localizes along newly synapsed axes (Fig. 3). As pachynema progresses, the number of MSH4 foci begin to drop. MLH1 foci become visible during a relatively short period (1 day) and colocalize with a subset of MSH4 foci (Santucci-Darmanin et al. 2000) and Fig. 3. In mouse oocytes, MLH1 foci appear during zygonema and persist until diplotene (Baker et al. 1996). MLH1 foci also first become apparent in human oocytes during zygonema and their number reaches a stable plateau by late zygonema (Lenzi

⁴ Dictyate stage: The dictyate stage of meiotic prophase is a prolonged resting phase that is terminated shortly before ovulation.

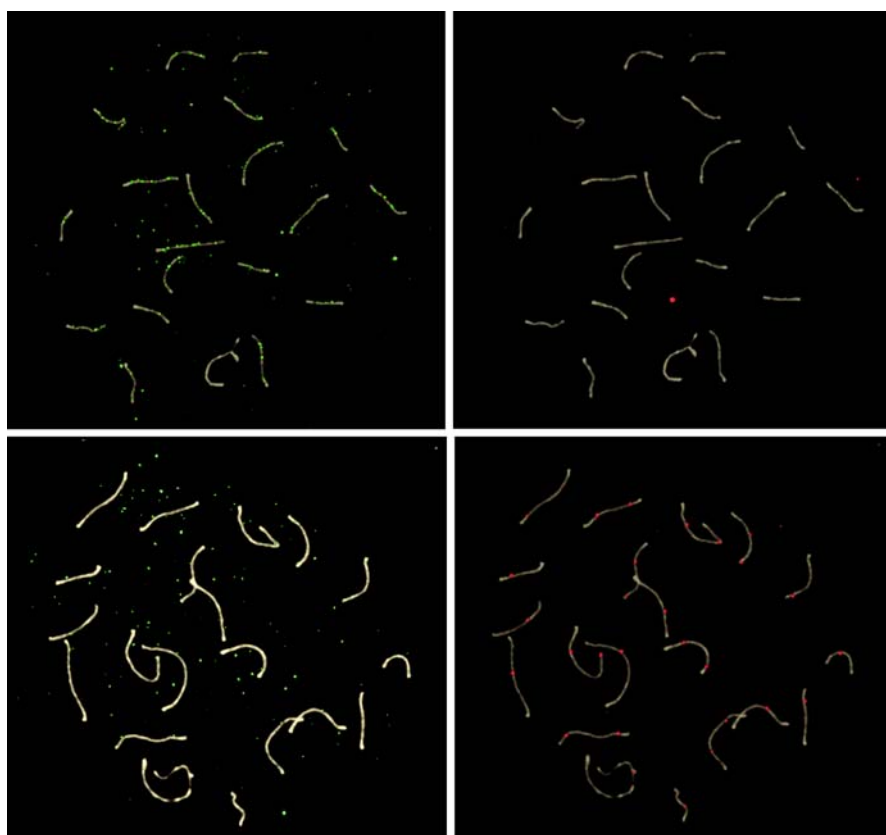


Fig. 3 Distribution of MSH4 (left) and MLH1 (right) on SCs in early (upper two panels) and mid- pachytene nuclei of mouse spermatocytes. As can be seen, MSH4 localizes at multiple sites along each SC during early pachynema. As the number of MSH4 foci drops during mid-pachynema, MLH1 foci colocalize with some of the remaining MSH4

et al. 2005), suggesting an earlier commitment to crossover in oocytes than spermatocytes.

As mentioned above, MLH1 forms a heterodimer with MLH3, another mismatch repair protein during meiosis (Lipkin et al. 2002). As is the case with *Mlh1*^{-/-} mice, *Mlh3*^{-/-} animals are sterile (Lipkin et al. 2002). The distribution pattern of MLH3 mimics that of MLH1 and, not surprisingly, the two proteins colocalize (Lipkin et al. 2002). Immunogold labeling provides conclusive proof that MLH3 is also a component of RNs (Lipkin et al. 2002). However, despite the fact that MLH1 and MLH3 are heterodimeric partners, MLH3 appears to be required for MLH1 localization (Kolas et al. 2005; Lipkin et al. 2002). For complete details of the labeling pattern and experiments that combine targeted disruption of each gene followed by immunolocalization of the other protein see Lipkin et al. (2002).

Cdk2, another important mitotic cell cycle protein, also colocalizes with MLH1 in addition to localizing to the ends (telomeres) of each SC (Ashley et al. 2001).

There have been numerous suggestions that a subset of SyNs may become RNs (Agarwal and Roeder 2000; Moens et al. 2002; Plug et al. 1998; Sherman and Stack 1995; Stack and Anderson 1986; von Wettstein et al. 1984; Zickler and Kleckner 1999). Consistent with this interpretation several SyN components are also RN components, a relationship that has been confirmed by co-localization of the SyN components with an antibody to MLH1. MLH1 has been shown to colocalize with a subset of both Rad51 and RPA foci (Plug et al. 1998) and with MSH4 (Santucci-Darmanin et al. 2000). However, perhaps for technical reasons, Moens et al. (2002) were unable to detect colocalization of RPA or BLM foci with MLH1 foci. In studies of appearance of these proteins relative to the progression of meiotic prophase, the number of foci of the protein components of the SyN complex (RPA, BLM, MSH4) appear to have already begun to drop before MLH1 foci are detected, suggesting that some of the processing of the DNA associated with the SyNs has already been completed in mouse spermatocytes by the time molecular events associated with reciprocal recombination have reached the stage of MLH1 and MLH3 involvement.

Given the fact that MLH1 foci have been shown to colocalize with RNs, one would expect a close correlation between the number of MLH1 foci and number of RNs for a species. This is not always the case. The number of RNs reported in human spermatocytes (Rasmussen and Holm 1978; Solari 1980) was considerably less than the reported number of chiasmata (Hultén 1974). However, the average number of 49.1 ± 4.8 MLH1 foci in spermatocytes (Lynn et al. 2002) and 70.3 (Tease et al. 2002) or 50.3 ± 24.7 MLH3 and 41.4 ± 26.5 MLH1 foci in oocytes (Lenzi et al. 2005) is much closer to the number of crossovers ($51\text{--}53$ male; ~ 88 female) estimated from the genetic linkage maps [for review see Lynn et al. (2004)]. Given the difficulties of identifying electron-dense “bars” in sections and differentiating between “nodules” and “bars”, it is easy to understand why recognizing a strong fluorescent signal on an equally strong fluorescently stained SC might provide a more accurate count. However, this explanation does not account for the discrepancy between the number of RNs vs. the number of MLH1 foci in spreads.

Yet in plants it is the RN count that corresponds more closely to the linkage data. Anderson et al. (2003) have generated high-resolution maps for *Zea mays* based on RN numbers and distributions. The average RN count per nucleus compares well with the observed number of chiasmata and the length of the genetic linkage map [see Sherman and Stack (1995)]. However, MLH1 counts in tomato identify only around two-thirds of the crossovers estimated from chiasmata or genetic linkage maps (de Boer et al. 2007). To date, an explanation for the apparent discrepancy has remained elusive, but see discussion below.

5

RN and MLH1 Mapping

Construction of a linkage map by genetic analysis requires multiple markers (genes with different alleles) and evaluation of large populations. Assuming all crossovers can be detected with an antibody such as one for MLH1, or by identification of chiasmata or RNs, cytological analysis has the advantage of allowing determination of the number and placement of all crossovers in a meiotic nucleus without the necessity for multiple genetic markers. Early cytological counts and maps were based on number of chiasmata and their position along a bivalent in diakinesis/metaphase I cells. The amount of compaction and technical difficulties in interpreting these configurations rendered many diakinesis/metaphase I cells unusable for analysis. In contrast, the chromatin in microspread preparations of pachytene nuclei is less condensed and RNs or MLH1 foci can be mapped more accurately. In addition, pachynema in mouse spermatocytes lasts 7.3 days vs. less than a day for diakinesis (Oakberg 1956) resulting in a higher percentage of usable spermatocytes for analysis. Similar ratios hold in other mammals. Since mammalian oocytes arrest at the dictyate stage until puberty, chiasmata analysis requires super-ovulation, a process that may induce artifacts. Moreover, chiasmata analysis from the resulting meiotic preparations is even more difficult than in spermatocytes (Hultén et al. 1978). Despite the fact that RN or MLH1 analysis necessitates use of fetal oocytes, far more data can be collected from these pachytene oocytes than from the super-ovulated mature oocytes (Lenzi et al. 2005).

As discussed above, determining the position of MLH1 sites along SCs has proven to be an excellent method of detecting and mapping sites of crossovers in both mice (Anderson et al. 1999; Froenicke et al. 2002; Koehler et al. 2002) and humans (Ashley et al. 2006; Saitta et al. 2004). By combining fluorescent antibody localization (FAL) procedures with fluorescent in situ hybridization (FISH) it is even possible to identify each bivalent within a complement. Following measurements, recombination maps can be constructed for each chromosome (Froenicke et al. 2002). The location of the MLH1 foci along the length of individual bivalents can be mapped and the data compartmentalized into bivalents with one MLH1 focus and those with two. The autosomal bivalents in mouse spermatocytes almost always have only one or two MLH1 foci per bivalent. There is a high frequency of MLH1 foci near the distal (non-centromeric) end of each bivalent, reflecting the high rate of recombination in male mice at these ends [for discussion see Froenicke et al. (2002)]. This study found that SC length is a better predictor of number of crossovers than is mitotic metaphase length (Froenicke et al. 2002). MLH1 maps have also been constructed for several of the autosomal bivalents in human spermatocytes and the correlation between SC length and crossover frequency appears to hold for entire nuclei (Lynn et al. 2002).

By combining maps of FISH-labeled DNA sequences of known genetic map position with RN (Anderson et al. 2005) or MLH1 (Ashley et al. 2006; Froenicke et al. 2002; Saitta et al. 2004) maps it is also possible to anchor the cytogenetic maps to the genetic linkage maps. Such efforts have advanced more rapidly in plants than in mammals. Anderson et al. (2005) used RN maps to construct a RN-cM map for maize. Based on this data Lawrence et al. (2006) used this relationship to map ESTs (Expressed Sequence Tags) throughout the maize genome. Efforts such as these are essential for integrating the genetic linkage and cytogenetic maps. It is likely that factors such as chromatin configuration influence crossover location and these can still be identified more easily by cytological techniques than by other methods [see Holmquist and Ashley (2006)].

6

Regulation of Number and Distribution of Crossovers per Bivalent

6.1

The Obligate Crossover

A single crossover is required to assure disjunction of the homologs to opposite poles at anaphase I. Given the restricted number of crossovers per nucleus and the random distribution of crossovers (and RNs) one would predict that some bivalents would have no crossovers. Moreover, if distribution were random, smaller chromosomes should be less likely to receive a crossover than larger ones. This is not the case. In species that have only one or two crossovers per bivalent, even the smallest bivalent virtually always receives a crossover. For example, birds have micro-chromosomes, yet each pair of homologs synapses and has an RN (Pigozzi and Solari 1999). Although in most mammalian species the Y chromosome is larger than bird micro-chromosomes, the actual region of homology (the pseudoautosomal region) between the X and Y is even less. For example, in humans the pseudoautosomal region of homology is 2.5 MB (Rouyer et al. 1986). Yet an RN is regularly observed (Rasmussen and Holm 1978; Solari 1980).

6.2

Crossover Interference

Throughout this discussion the focus has been on cytological aspects of meiosis. However, crossover interference was first described by early *Drosophila* geneticists as a genetic phenomenon. Both Sturtevant (1915) and Müller (1916) noted crossovers in *Drosophila* were non-randomly distributed, with closely spaced double-crossovers (two exchanges between closely linked genes) occurring much less frequently than expected by chance. Both called

the phenomenon crossover interference. RN or MLH1 maps are an excellent method of studying crossover interference at a cytological level.

However, recent evidence from *S. cerevisiae* suggests there may be two crossover pathways in this species: those that are subject to crossover interference and those that are not. Those crossovers not subject to interference utilize a pathway that does not appear to involve Holliday junctions (Osman et al. 2003), but does utilize MMS4⁵ and MUS81 [see review by Hollingsworth and Brill (2004).].

A noninterference pathway may explain an apparent deficiency in the mouse data. When the MLH1 counts in mouse spermatocytes are converted to centimorgans (cMs), the estimated total map length is 1195.4 cM (Froenicke et al. 2002). This estimate is somewhat shorter than the genetic maps based on molecular markers: 1361.2 cM (Dietrich et al. 1996) and 1530 cM (Blake et al. 2002). Although Froenicke et al. (2002) discussed this discrepancy in terms of either an under-estimate of the number of MLH1 foci or an over-estimate of the genetic map length, the discovery of a noninterference pathway merits a re-examination of the data. Guillon et al. (2005) found that MLH1 is required for only 90–95% of the crossovers in mouse and suggested that the remaining 5–10% of crossovers occurred via the noninterference pathway. Since the MLH1 map of Froenicke et al. (2002) accounts for only 88% of the crossovers in the Dietrich et al. (1996) map and only 78% of the crossovers in the Blake et al. (2002) map, the estimated 5–10% noninterference crossovers could theoretically account for most of the discrepancy. The existence of a noninterference pathway has indeed been reported (deBoer et al. 2006). The existence of a noninterference pathway raises the question: “Why is there crossover interference?” Until now, the answer has been “to assure that a second crossover does not occur in ‘too close’ proximity to the first”. What exempts a subset of crossovers from this constraint?

As discussed above, there is a significant discrepancy between the number of RNs and number of MLH1 foci observed in plant microsporocytes (Lhuissier et al. 2007). Might plants, like yeast, utilize a second crossover pathway that is not subject to interference? Analysis of *Mre3* and *Msh4* homologs in *Arabidopsis* suggests that plants may indeed have an interference and a noninterference crossover pathway (Mercier et al. 2005) (see also, G. Jones and C. Franklin, in this BOOK). MLH1 is thought to interact with Holliday junctions, that may not be involved in the noninterference pathway (Osman et al. 2003). If the noninterference pathway involved RNs but not MLH1, the “shortage” of MLH1 foci might be explicable. Arguing against this explanation, the RN distribution patterns in maize and tomato clearly exhibit crossover interference, a pattern that should be obscured if noninterference events are included.

⁵ MMS4: subunit of the structure-specific Mms4p-Mus81p endonuclease.

There are numerous suggestions that the interference signal is transmitted along the SC [for discussion, see Lynn et al. (2004); Roeder (1990); Zickler et al. (1992)]. Consistent with this interpretation, species that do not form SCs, such as the unicellular fungi, *Aspergillus nidulans* and *Schizosaccharomyces pombe*, do not form SCs, nor do they exhibit any crossover interference (for review, see Egel 1995).

A link between initiation of SC formation and interference has been noted in several organisms but the details may differ between species. In *Sordaria* a series of mutants result in modifications in interference pattern of RNs (distance between) and these changes are correlated with the differences in the initiation and extension of the SC at zygotene. This led Zickler et al. (1992) to state "Interference and nodule localization is an indirect expression of pairing pattern, at least in *Sordaria macrospora*." In *S. cerevisiae* the axial association sites revealed in the *zip1* mutant are both sites of synaptic initiation and the location of those crossovers that will exhibit interferences.

The recombination pattern measured by RNs in tomato (Stack and Anderson 1986) and maize also reflects the synaptic initiation pattern (Stack and Anderson 2002). Stack and Anderson (1986, 2002) noted that early nodules (SyNs) appear to assemble at pairing forks and, since synapsis in maize generally begins near the ends, they suggested that these distal SyNs are ready to begin the molecular steps leading to crossovers earlier than those assembled later (Stack and Anderson 2002). Therefore, when a SyN is successful at achieving a crossover (or some critical step in the process), an interference signal from the crossover site suppresses recombination activity in nearby SyNs.

A general correlation between synaptic initiation and crossover distributions have been made in mouse (Anderson et al. 1999; Froenicke et al. 2002) and human (Brown et al. 2005) spermatocytes. In the later study, preliminary experiments suggest that synapsis in spermatocytes most often begins near chromosome ends where there is a high frequency of crossovers in eutherian males (Brown et al. 2005).

The correlation between crossover interference and the presence of an SC returns us to the issue: "When do DSBs occur?" It appears that in order to regulate number and distribution of crossovers the interference mechanism must be able, not only to detect a DSB, but once a commitment to a crossover has been made, be able to shunt additional repair into either the "gene conversion" or the "noninterference" pathway. If the SC is involved in transmission of the interference signal, this scenario would suggest that DSBs cannot be made before the SC is formed. The presence of Rad51 in leptotema/early zygonema in mammalian spermatocytes does not negate this model, if it is present at gaps between ssDNA and dsDNA at stalled replication forks.

7

Summary and Final Comments

This chapter has raised questions about the following commonly held assumptions about meiosis:

1. Mechanisms of synapsis and crossing over and their control are universal. If a mechanism (or pathway) has been proposed in one species, then it is unwise to assume that the mechanism (or pathway) operates in the same manner in distantly related taxa. Different species have different meiotic requirements and are likely to have evolved entire pathways and systems to best serve those needs. Some of these approaches appear to involve meiotic-specific proteins that best serve the specific needs of the species; others are likely to be meiotic adaptations of changes in chromatin structure and regulatory mechanisms, including cell cycle control that have evolved in concert with regulatory changes in somatic cells.
2. Once a function has been assigned a protein, it cannot be assumed that it plays that role, and that role only, in other taxa. Proteins play multiple roles under different circumstances and evolve additional functions in different species.

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Human Recombination Hotspots: Before and After the HapMap Project

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Abstract Due to the inherent difficulties of studying recombination in humans, meiotic recombination hotspots have been best characterised in model organisms such as *Saccharomyces cerevisiae*. However, several intriguing features of human recombination have been unveiled by new analytical methods, including significant differences in both crossover rate and distribution between individuals and across chromosomes. Furthermore, studies at the highest resolution have shown that human meiotic crossovers generally concentrate into local hotspots that separate the genome into a series of relatively recombinationally inert haplotype blocks. The HapMap project has taken advantage of this pattern of recombination and is characterising the haplotype structure of the entire human genome, primarily to aid genome-wide association studies of complex, common disease. HapMap has also provided a tool for population geneticists to investigate genome-wide patterns of historical recombination at varying levels of resolution. However, it is only from the direct, high-resolution analysis of recombination events that we can truly appreciate what is currently happening at specific hotspots, and gain valuable clues about the dynamics and mechanisms of human meiotic recombination, the evolution of hotspots and their effect on genome diversity and evolution.

Keywords Crossover · Gene conversion · Hotspots · Linkage maps · HapMap project · Sperm analysis · Ectopic recombination

Abbreviations

cM	CentiMorgan
CNV	Copy number variation
DSB	Double-strand break
DSBR	Double-strand break repair
HNPP	Hereditary neuropathy with liability to pressure palsies
LCR	Low copy repeat
LD	Linkage disequilibrium
LDU	Linkage disequilibrium unit
LE	Linkage equilibrium
LINE	Long interspersed nuclear element
MEP	Minimal efficient processing segment
MHC	Major histocompatibility complex
NAHR	Non-allelic homologous recombination
PAR1	Major pseudoautosomal region at the ends of Xp/Yp
PSV	Paralogous sequence variant
RF	Recombination fraction

RN	Recombination nodule
SINE	Short interspersed nuclear element
SNP	Single nucleotide polymorphism
TF	Transcription factor

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Introduction

Gaining a thorough understanding of meiotic recombination in our own species has wide-reaching consequences. Knowing exactly how recombination influences patterns of sequence diversity will ultimately help us to understand the origins of human populations and the dynamics of human DNA evolution (Pääbo 2003) and should aid the design of genetic association studies to identify susceptibility loci for complex diseases (Jorde 2000). Furthermore, the study of aberrant recombination events, such as unequal crossover, should provide insights into pathological genome rearrangements frequently seen in inherited disorders and cancer (e.g. Higgs et al. 1989; Pentao et al. 1992; Feunteun 1998; Lupski and Stankiewicz 2005).

It has long been recognised that there is no simple relationship, other than colinearity, between genetic and physical maps in a wide variety of organisms, and that regions of both high and low meiotic recombination exist at varying levels of resolution (Lichten and Goldman 1995; Wahls 1998). Intense and highly localised recombination intervals, known as recombination hotspots,¹ have been well characterised in the yeast *Saccharomyces cerevisiae*, in which analyses are greatly helped by the ability to recover all four products of a single meiosis. Hotspots preferentially occur in intergenic regions corresponding to gene promoters (Cao et al. 1990; Baudat and Nicolas 1997) and show meiosis-specific DNaseI or micrococcal nuclease (MNase) sensitivity (Fan and Petes 1996; Ohta et al. 1994; Wu and Lichten 1994), indicating the presence of open chromatin domains. Yeast hotspots can be classified into three types. Those whose activity depends on *trans*-acting transcription factors (TFs) (White et al. 1991, 1993), but not transcription itself (White et al. 1992), have been termed α hotspots and are thought to be common in eukaryotes (Kirkpatrick et al. 1999a). Variation in α hotspot activity may reflect the varying ability of different TFs to efficiently recruit the Spo11 endonuclease that initiates recombination by introducing a double-strand DNA break (DSB) (Keeney et al. 1997; Keeney and Neale 2006). This may explain why not all TF-binding regions in yeast represent hotspots (Baudat and Nicolas 1997). The second class of hotspots, known as β hotspots, are associated with

¹ We use the term hotspot to denote a *local clustering* of recombination, such that the *relative frequency* of exchange is higher within a hotspot than is observed in the immediate flanking DNA. Biologically speaking, this is more meaningful than defining hotspots as regions with a higher than average genome-wide recombination rate.

cis-acting nucleosome-excluding DNA sequences which stimulate both meiotic recombination and gene expression but to which no TF is known to bind (Cao et al. 1990; Rattray and Symington 1993; Wu and Lichten 1995; Xu and Kleckner 1995; Wang and Griffith 1996; Kirkpatrick et al. 1999a,b). Significant associations of hotspots with regions of high GC base composition have also been observed (Gerton et al. 2000) and these have been termed γ hotspots (Petes 2001).

Until recently, our understanding of human hotspots has in comparison been rather rudimentary. Cytogenetic and linkage studies in families have provided a low-resolution, genome-wide picture and highlighted the existence of recombinationally hot domains if not hotspots per se. The latter can only be properly defined by high-resolution sperm DNA approaches and even now only around 20 have been characterised directly. However, recent large-scale genomic projects, specifically the International HapMap Project and allied projects, have provided extensive information on DNA diversity that has allowed population geneticists to gain an unprecedented view of historical recombination activity throughout our genome. Here we discuss how our knowledge has been refined in recent years by these varied and complementary approaches.

2

Before the HapMap Project

2.1

Low-Resolution Studies

Classically, meiotic recombination has been investigated directly in human spermatocytes by using chiasmata to determine the numbers and distributions of crossovers at diakinesis or metaphase I (Hultén 1974). More recently alternative, immunocytogenetic approaches have been developed to exploit the behaviour of certain associated proteins. In particular, the mismatch repair protein MLH1, a component of late recombination nodules (RNs) (see the chapter by Ashley, this volume), forms discrete foci along the axes of homologous chromosomes at the pachytene stage of meiosis, providing a method for visualising sites of recombination with a somewhat higher degree of resolution (Baker et al. 1996; Barlow and Hultén 1998; Anderson et al. 2001; Sun et al. 2006). For full review of all the cytogenetic approaches to the study of recombination, see Hultén et al. (2005).

These cytogenetic analyses have shown that there has to be at least one crossover per chromosome to ensure proper chromosome segregation. This phenomenon is known as obligate chiasmata. Beyond this, the number of crossovers per kilobase increases with decreasing chromosome size, rather than remaining constant as would be expected if crossovers occurred ran-

domly (Hultén 1974; Jones 1984; Kaback et al. 1989, 1996). Meiotic recombination hotspots likely contribute to these observations, given the fact that, in *S. cerevisiae*, both the density and the recombinational activity of hotspots on smaller chromosomes is significantly greater than on the larger chromosomes (Gerton et al. 2000).

In humans, chiasmata can arise at any position along an autosomal bivalent, although they do show significant preferences controlled in part by crossover interference, in which a crossover at one locus decreases the probability of another crossover in the vicinity (Hultén 1974; Laurie and Hultén 1985). Indeed, mutations that reduce interference in *S. cerevisiae* also randomise the distribution of crossovers among chromosomes, with the result that homologous pairs sometimes fail to crossover and as a result undergo nondisjunction (Egel 1995; Sym and Roeder 1995; Chua and Roeder 1997). Major features such as heterochromatin and centromeres also appear to influence the positions favoured for chiasma formation in humans (Laurie et al. 1981; Saadallah and Hultén 1983; Goldman and Hultén 1993). In male meiosis, chiasma form preferentially in telomeric and subtelomeric regions (Hultén 1974; Laurie and Hultén 1985), a pattern mirrored by the distribution of MLH1 foci in pachytene spermatocytes (Barlow and Hultén 1998). In contrast, crossovers tend to be more interstitial in the limited number of oocytes analysed (Wallace and Hultén 1985; Hultén et al. 1990; Tease et al. 2002).

Linkage maps of the human genome, originally based on the inheritance pattern of a few hundred polymorphic loci in a small number of three-generation families, have also shown large-scale variation in crossover distribution (e.g. Donis-Keller et al. 1987; NIH/CEPH Collaborative Mapping Group 1992; Weissenbach et al. 1992; Gyapay et al. 1994; Broman et al. 1998; Mohrenweiser et al. 1998). Moreover, some studies have suggested positive correlations between recombination rate and DNA sequence features. For example, Majewski and Ott (2000) proposed a link between GT microsatellites and regions of high recombination on chromosome 22. Similarly, a small but significant amount of local variation in recombination rate might be explained by GC content (Yu et al. 2001), although closer inspection shows that regions with a high CpG fraction but low GC and poly(A/T) content tend to have the highest recombination rates (Kong et al. 2002).

Human linkage maps have also highlighted considerable gender differences in recombination rates with average female recombination rates, even across individual chromosomes, being significantly higher than those in males. The most extreme example is on the sex chromosomes. The possibility of recombination between the mammalian X and Y chromosomes was first proposed in the 1930s (Koller and Darlington 1934), but it was over 50 years later that linkage maps explicitly demonstrated that crossover between the 2.7-Mb pseudoautosomal pairing region PAR1 at the ends of human Xp and Yp was an obligatory event in male meiosis (Cooke et al. 1985; Simmler et al. 1985; Rouyer et al. 1986). Recombination between X and Y is almost entirely

restricted to PAR1 and to a lesser extent PAR2, the 0.33-Mb pseudoautosomal region at the tips of the long arms of the X and Y chromosomes (Frieje et al. 1992), whereas in the female germline the two X chromosomes can recombine anywhere along their entire length. This results in marked sex-specific differences in PAR1 genetic map length, which is 50 cM in males and around one tenth of this in females (Rouyer et al. 1986; Page et al. 1987). PAR1 is thus a male-specific recombination hot domain with a mean crossover frequency 20 times higher than the genome average (Rappold 1993).

The human genome sequencing project (International Human Genome Sequencing Consortium 2001; Venter et al. 2001) has allowed the comparison of genetic linkage maps with detailed physical maps, allowing analysis of rates of crossover per unit distance of DNA (Yu et al. 2001; Kong et al. 2002). The Yu et al. study, based on 188 meioses, showed that the average female recombination rate was 1.68 cM/Mb, nearly double the average male rate of 0.92 cM/Mb. Regionally, recombination rates can vary from 0 to 8.8 cM/Mb, with intervals of up to 6 Mb in length with particularly low or high recombination rates that have been termed recombination deserts and jungles, respectively. As suggested by cytogenetic analyses, this sex-specific variation is actually rather complex; male-specific rates of recombination are higher than female rates in subtelomeric regions, and there are other regions, near centromeres for example, where the reverse is true (Broman et al. 1998; Mohrenweiser et al. 1998; Kong et al. 2002). Consistent with cytogenetic surveys, genetic maps also show that shorter chromosomes have higher recombination rates per megabase than longer ones, with for example chromosomes 21 and 22 showing twice the rate of chromosomes 1 and 2 (Kong et al. 2002).

Kong et al. (2002) improved the resolution of the Yu et al. (2001) map fivefold by analysing 1257 meioses in 146 two-generation Icelandic families using 5136 microsatellite markers. Minor differences between the maps, for instance a slightly lower sex-averaged recombination rate of 1.13 cM/Mb in the Iceland map, can be attributed to the higher resolution of this map as well as the use of different drafts of the human genome sequence. Genetic linkage maps have recently been constructed in four population groups (African Americans, Mexican Americans, East Asians and whites) to assess ethnic variation in map length (Jorgenson et al. 2005). The maps for each population actually proved very similar, with only minor variation such as a slight overall expansion of the African American map and some occasional local ethnic differences, for instance in a small region of chromosome 8p.

Both cytogenetic studies and linkage maps have shown that recombination rates can vary significantly between females (Palmer and Hultén 1983; Laurie and Hultén 1985; Broman et al. 1998; Lynn et al. 2002; Kong et al. 2002; Tease and Hultén 2004; Hultén et al. 2005; Sun et al. 2006) and a recent huge survey of 14 140 meioses revealed a significant positive correlation between maternal recombination rate and maternal age (Kong et al. 2004). The authors suggested that recombination rates do not actually increase with age but instead

that selection increases the chance of an egg with more crossovers producing a successful live birth. This is consistent with reduced levels of recombination and increased maternal nondisjunction being associated with maternal age (reviewed within Hassold et al. 2000) and, as a result, increased meiotic recombination may protect against certain nondisjunction events (Kong et al. 2004). Linkage map surveys have yet to identify inter-individual variation in paternal recombination rates but studies in spermatocytes have shown that the frequency of chiasmata and of MLH1 foci can show significant variation between men, albeit at a much lower level than that seen between women (Palmer and Hultén 1983; Laurie and Hultén 1985; Lynn et al. 2002; Tease and Hultén 2004; Hultén et al. 2005; Sun et al. 2006). The apparent lack of this variation in live births has led to the suggestion that selection might also take place in the male germline, at the level of spermatocytes (Kong et al. 2004).

Overall, similar conclusions can be drawn from cytogenetic and linkage map analyses, but their limited resolution means that they can reveal nothing about fine-scale crossover activity along chromosomes.

2.2

Improving the Resolution

2.2.1

Linkage Disequilibrium Studies

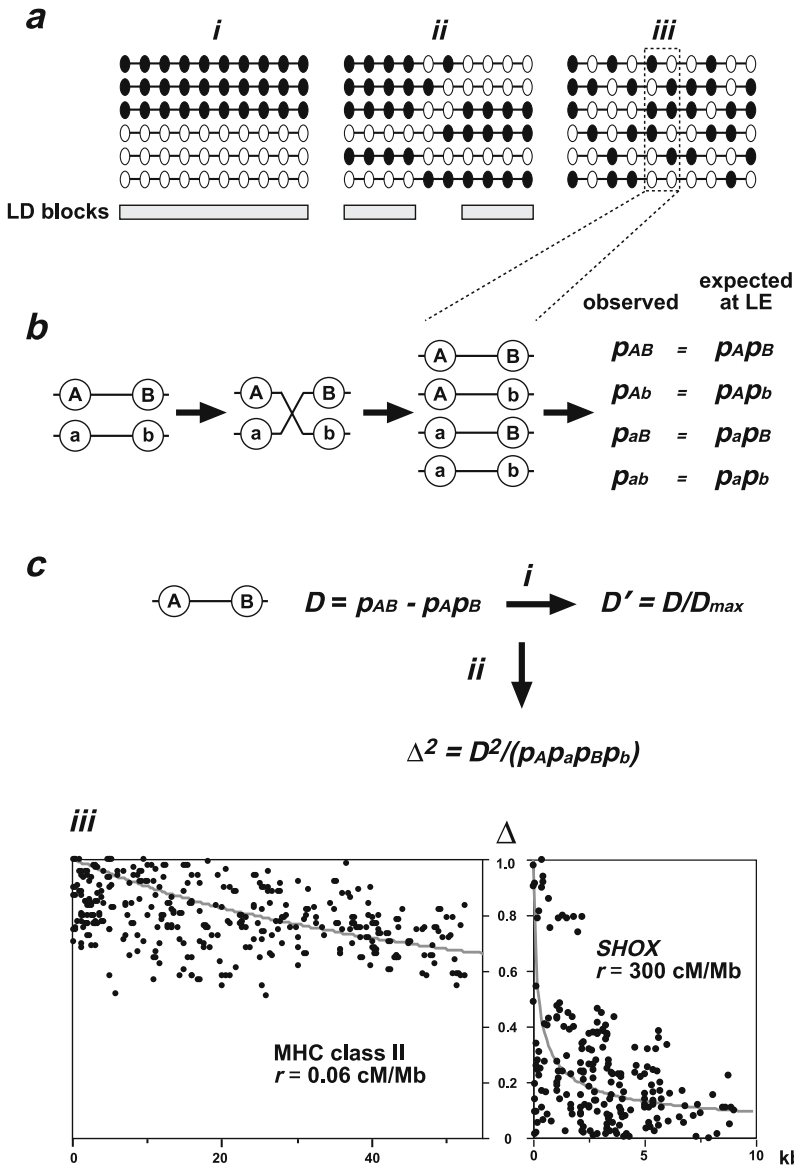
The small size of human families and the low frequency of crossover events per unit length of DNA impose a resolution limit of 0.1–1.0 cM (typically 0.1–1.0 Mb) on crossovers detected in pedigrees. Considerably improved resolution can be achieved through analyses of patterns of linkage disequilibrium (LD), which is the non-random association of alleles at closely linked loci (Fig. 1a). In general, LD decays as distance increases between markers. The main force that breaks down LD is recombination, so a genomic region in intense LD is likely to have been recombinationally inactive during the history of the population; alternatively, crossovers may have arisen historically but failed to leave descendants in the contemporary population. LD studies of high densities of single nucleotide polymorphism (SNP) markers allow a much higher resolution, albeit indirect, analysis of crossover than can be achieved through family studies, through the inference from haplotype diversity of the recombination events that have accumulated over thousands of generations.

Although it is a relatively simple concept, a satisfactory single measure of LD has yet to be developed. Most measures of LD capture the strength of association between a pair of bi-allelic sites and the most widely used pairwise measures of LD are $|D'|$ (Lewontin 1988) and Δ^2 (more commonly known as r^2) (reviewed in Devlin and Risch 1995). Both are based on the difference between the observed frequency of a haplotype and the frequency expected

under free association from the product of allele frequencies (Fig. 1). The case of $|D'| = 1$ is known as complete LD and will only occur if two bi-allelic loci show only two or three of the four possible haplotypes, implying lack of historical recombination and no recurrent mutation. Free association of loci is indicated when $|D'| = 0$, and values of $|D'| < 1$ suggest incomplete disruption of the complete ancestral LD. Absolute association (perfect LD) is indicated by $\Delta^2 = 1$, which cannot occur unless the allele frequencies at both loci are equal and only two of the possible four haplotypes exist in a population.

The Δ^2 measure has several properties that make it more useful than $|D'|$. For example, under the standard neutral model of molecular evolution (Kimura and Crow 1964; Kimura 1968, 1985), population genetics theory provides a simple relationship between Δ^2 , the effective population size (N_e), the recombination rate per unit distance (r) and inter-marker distance (d) as $\Delta^2 = 1/(1 + 4N_e r d)$ (Sved 1971). Furthermore, in contrast to $|D'|$, where values between 0 and 1 have no clear interpretation (Ardlie et al. 2002) and are thus of little use for comparing levels of LD between studies, intermediate values of Δ^2 are of considerable use as they are inversely proportional to the sample size required to detect statistically significant LD between two loci. Hence, a Δ^2 value is related to the amount of information provided by one locus about the other (Kruglyak 1999; Pritchard and Przeworski 2001; Weiss and Clark 2002). In addition, $|D'|$ is artificially inflated in small samples while Δ^2 shows much less bias upwards. However, due to its dependency on allele frequency, Δ^2 is typically lower than $|D'|$ for any chromosomal interval (Weiss and Clark 2002) and it is important to note that measures of $\Delta^2 < 1$ can occur even without recombination, while values of $|D'| < 1$ do signal historical recombination events. [More recently, additive LD units (LDU) have been derived for association mapping purposes, see Sect. 3.1.].

Several small-scale surveys of LD have led to the identification of a number of putative human meiotic recombination hotspots inferred from regions of local LD breakdown. These include hotspots in the β -globin gene cluster (Chakravarti et al. 1984), near the human insulin gene (Chakravarti et al. 1986), within the major histocompatibility complex (MHC) class II region (van Endert et al. 1992; Carrington et al. 1994) and at the *PGM1* gene (Yip et al. 1999; Rana et al. 2004). However, patterns of LD can be influenced not only by recombination rate but also by recurrent mutation and demographic processes such as natural selection, genetic drift, population bottlenecks and admixture. Inferences of recombination hotspots from LD should therefore be treated with caution (Hedrick 1987; Ardlie et al. 2002). Additional studies have shown considerable variation in the extent of LD from one genomic region to another (Taillon-Miller et al. 2000; Abecasis et al. 2001; Reich et al. 2001, 2002). Variation in LD patterns also occurs between populations (Laan and Pääbo 1997; Frisse et al. 2001; Reich et al. 2001), as exemplified by the consistent observation that LD in non-African populations extends over longer distances than in Africans, as a consequence of the non-African popu-



lations having passed through more genetic bottlenecks (Gabriel et al. 2002). Therefore, the interpretation of LD studies depends on understanding the rules that govern LD patterns in the human genome and requires validation by direct kilobase-scale high-resolution analysis of crossover events; the latter is only possible by sperm typing.

- ◀ **Fig. 1** Measures of linkage disequilibrium. **a** Linkage disequilibrium (LD) is the non-random association of alleles at closely linked loci. (i) Strong association between all markers in a given region suggests that those markers are part of an LD block in which recombination has been suppressed historically. (ii) Free association between LD blocks implies clustering of historical recombination (Sect. 2.3 and Fig. 2). (iii) Lack of any association or LD between any markers implies that the region has been recombinationally very active. **b** Consider two loci, one with alleles A, a and the other with alleles B, b. At linkage equilibrium (LE) caused by meiotic recombination, all four of the possible two-locus haplotypes will be observed at frequencies expected from allele frequencies. Thus at equilibrium, the frequency p_{AB} of haplotype AB will be $p_A p_B$, where p_A and p_B are the frequencies of alleles A and B. **c** The simplest measure of LD is the difference between the observed and the expected frequencies of a haplotype, e.g. $D = p_{AB} - p_A p_B$. (i) To avoid dependence on allele frequencies, D can be scaled as $D' = D/D_{\max}$, where $D_{\max}$ is the lesser of $p_A p_B$ or $p_a p_b$ if D is negative, or $p_a p_B$ or $p_A p_b$ if D is positive. D' can be positive or negative depending on the arbitrary labelling of alleles, and the absolute value $|D'|$ is normally used. A $|D'|$ value of 1 indicates complete LD and thus no evidence for historical recombination between the loci, while $|D'| = 0$ indicates complete LE. (ii) Δ^2 is another scaling of D and provides the statistical correlation between alleles at two sites. It is obtained by dividing D^2 by the product of the allele frequencies at the two loci; in this example, $\Delta^2 = D^2 / (p_A p_a p_B p_b)$. Δ^2 can take values from 0 (complete LE) to 1 (absolute LD, only two haplotypes present). The relationship $\Delta^2 = 1 / (1 + 4N_e r d)$ (see main text) provides a method for comparing the extent of LD in different population surveys. (iii) Examples are shown of the relationship between Δ and the physical distance between pairs of SNP markers across a 52-kb haplotype block in the MHC class II region on chromosome 6 (Jeffreys et al. 2001) and across a 10-kb interval in the *SHOX* region within PAR1 at the end of Xp/Yp (May et al. 2002). Grey lines show best-fit values of the sex-averaged population recombination rate per unit distance, assuming N_e to be 10 000

2.2.2

Single Sperm Typing

In contrast to pedigree analyses, in which data from different families must be pooled to accumulate useful numbers of crossovers, the huge number of sperm available from any male (typically 10^8 sperm per ejaculate) offers the possibility of very high resolution analysis as well as studies of recombination activity at the level of individual men (Li et al. 1988). Classic sperm typing involves the separation of single sperm cells from a suitably informative man with sufficient heterozygosities at polymorphic markers across the region of interest, followed by whole-genome amplification of each cell using degenerate PCR primers. This routinely amplifies about 78% of the genome to 30–60 copies made up of fragments 300–600 bp in length (Zhang et al. 1992). Multiple loci from each cell can then be PCR amplified and typed to detect crossovers and to map exchange points within the chosen interval (for reviews, see Arnheim et al. 2003; Carrington and Cullen 2004). Single sperm typing has led to the identification of a putative recombination hotspot in a 280-kb-long DNA segment near the Huntington disease locus (Hubert et al. 1994). More recently, it has been used to identify modest local

variations in recombination rate across the pseudoautosomal region PAR1 (Lien et al. 2000) and to produce a high-resolution crossover map across the MHC (Cullen et al. 2002). Single sperm typing has also shown that significant decreases in recombination frequency in the PAR1 can lead to nondisjunction and thus the Klinefelter syndrome (XXY) (Shi et al. 2001). However, the resolution of most single sperm typing studies has been constrained by low marker densities and more importantly by the limited number of sperm typed, typically in the region of a few hundred per study.

2.3

High-Resolution Sperm Typing

The full analysis of human meiotic recombination, including the proper definition of crossover hotspots, requires the detection of exchange events in a chosen region of DNA that arise at much lower frequencies than have been detected by single sperm typing. An alternative method has therefore been developed that allows large batches of sperm DNA, rather than single sperm, to be interrogated for recombinant DNA molecules. This method is capable of giving a resolution of 0.0001 cM or less. The choice of genomic interval for study can be guided by initial LD analyses to gain clues about likely patterns of historical recombination. LD analysis can lead to one of several results (Fig. 1a). First, all markers in the interval may be in intense LD, suggesting that the whole region is likely to be recombinationally inert. Second, a slow and uniform decline of LD with physical distance would suggest that recombination events have occurred randomly across the region. Third, there may be regions of intense LD separated by an interval of free association; this sudden breakdown of LD implies the possible existence of a localised recombination hotspot. Fourth, lack of LD even between markers that are physically very close would suggest extreme recombination activity over the whole region. It is worth re-emphasising that LD studies only allow inference of historical recombination events. For instance, LD blocks can be generated through chance clustering of a few historical crossovers, without the need to invoke hotspot activity (Wang et al. 2002). A picture of what is truly happening can only be gained through the second step of this strategy, in which allele-specific PCR directed to heterozygous SNPs flanking a suitable test interval is used to recover crossover molecules directly from multiple aliquots of sperm DNA (Fig. 2). These methods were initially developed to explore the relationship between meiotic crossover and tandem repeat DNA instability in the germline (Jeffreys et al. 1998b). Indeed, the first human crossover hotspot to be defined at the molecular level was identified through the detection and analysis of crossovers at the MS32 minisatellite (Jeffreys et al. 1998a), and it was the discovery of this hotspot that provided the first clear indications that human meiotic crossovers may generally concentrate into very narrow (1–2 kb) hotspots interspersed with recombinationally inert DNA.

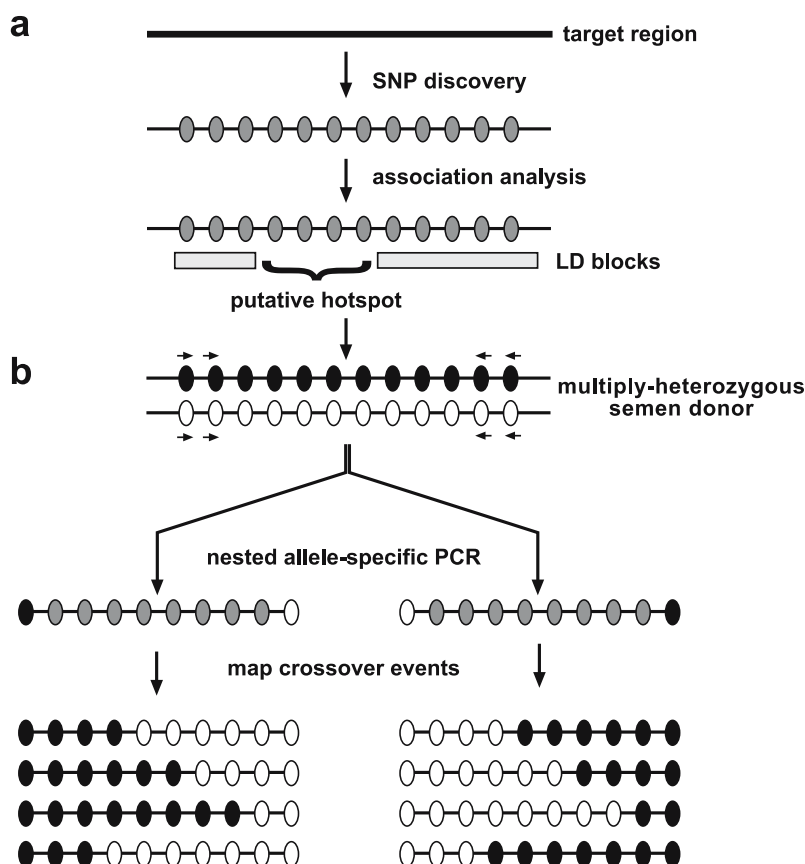


Fig. 2 A two-tier strategy for high-resolution analysis of meiotic crossover hotspots in human DNA. **a** A chosen target region is subjected to high-density SNP discovery, followed by genotyping in a panel of semen donors. SNPs are the marker of choice because of their high abundance, their low rate of recurrent mutation and their utility for physical selection of crossover molecules. LD patterns so revealed give clues about historical recombination events, for example the presence of a putative hotspot. **b** The second stage begins with identifying a suitably informative semen donor. Two rounds of repulsion-phase allele-specific PCR are used to selectively amplify recombinant molecules from batches of sperm DNA and crossover breakpoints are mapped by typing internal SNPs. Reciprocal crossovers (*black-white*, *white-black*) can be recovered separately using appropriate primer combinations

The first test of the two-tier high-resolution strategy was directed towards a putative hotspot in the MHC class II region (Jeffreys et al. 2000). Haplotyping had already revealed domains of LD in free association across a 15-kb interval between the *TAP1* and *TAP2* genes (van Endert et al. 1992; Carrington et al. 1994) and, within this interval, two maternal crossovers detected in families had been shown to co-localise to an 850-bp interval within the second

intron of *TAP2* (Cullen et al. 1995). However, it remained possible that this co-localization could have arisen by chance and that the whole 15-kb interval merely showed modest recombinational enhancement (Jeffreys et al. 2000). Sperm crossover analysis did, however, reveal a genuine hotspot ~ 1.2 kb wide and of modest recombinational activity in sperm (peak ~ 8 cM/Mb). One, and probably both, of the maternal crossovers localised by Cullen et al. (1995) map within this hotspot, suggesting that it functions in both male and female meiosis. The large-scale sexual dimorphism seen in recombination rates is also apparent at this much finer scale as population, pedigree and sperm data together suggest that the female recombination rate at the *TAP2* hotspot is very approximately 30-fold higher than that in males (Jeffreys et al. 2000).

This two-tiered analysis was then extended to a 240-kb segment extending upstream of *TAP2* (Jeffreys et al. 2001). This identified a further five human sperm crossover hotspots, all predictable from localised intervals of LD breakdown, in which about 95% of crossovers in the region occurred. The hotspots are not randomly distributed, but fall into clusters separated by blocks of LD that are 40 to 90 kb long, with 1–7 kb separating each hotspot within a cluster. This study was one of the first to provide convincing evidence that recombination hotspots strongly influence patterns of LD and that structuring of diversity into LD blocks is common in the human genome.

Although the concordance of LD breakdown and the locations of sperm crossover hotspots strongly suggest that the same hotspots function in female meiosis, direct high-resolution analysis of these hotspots in oocytes is not feasible. This impedes the quantitative analysis of the relationship between crossover distribution and LD patterns. One solution, therefore, is to target genomic regions known to be particularly proficient in male recombination, the classic example being PAR1. Analysis of a 43-kb interval around the *SHOX* gene, approximately 550 kb from the PAR1 telomere (Rao et al. 1997), revealed extremely rapid decay of LD with physical distance, with significant LD extending only a few kilobases at most, as expected for a region very active in (male) recombination. However, sperm crossover analysis in a 9.9-kb region within this interval, showing evidence for LD block structure and local LD breakdown, revealed a highly localised hotspot about 2 kb wide flanked by recombinationally much less active DNA (May et al. 2002). This hotspot shows a peak activity of 250 cM/Mb, making it the most active hotspot yet defined by sperm typing, but was otherwise very similar to autosomal hotspots. This suggests that hotspots also play a role in crossover distribution in PAR1 but, even outside the hotspot, there is sufficient recombination to cause a rapid decay of LD.

All human crossover hotspots characterised to date share very similar features. All show sperm crossover resolution points smoothly and symmetrically distributed across the hotspot, with exchanges approximately normally distributed. Only one hotspot to date shows a skewed crossover distribu-

tion, possibly caused by an AT-rich palindromic minisatellite located within the hotspot that might perturb processing of recombination intermediates (Jeffreys and Neumann 2005). Depending on marker density, the centres of hotspots can be mapped quite precisely, to within ± 30 bp. Crossover hotspots all show a constant width of 1–2 kb within which 95% of crossovers occur. The great majority of crossovers are simple, with a switch from one haplotype to the other in a single interval between informative SNPs. Only about 1% of crossovers are more complex, showing a patchwork of DNA from both haplotypes at the site of crossover indicating patchy gene conversion occasionally accompanying crossover. Most hotspots show fully reciprocal exchange in terms both of rate and of sites of crossover resolution. In contrast, the peak intensity over different hotspots varies considerably, from 0.4 cM/Mb for the *DNAI* hotspot in the MHC class II region to 250 cM/Mb for the *SHOX* hotspot (Jeffreys et al. 1998a, 2001; May et al. 2002). In contrast to nearly all yeast recombination hotspots that are associated with transcriptional promoter regions (Cao et al. 1990; Baudat and Nicolas 1997), only one of these human hotspots (incidentally the weakest yet discovered) is similarly located. The others are found in a wide variety of inter- and intra-genic locations and none shares any obvious primary sequence similarity (Jeffreys et al. 2004).

3

The HapMap Project

The International HapMap project was launched in October 2002 with the primary goal of establishing a freely available SNP haplotype map of the human genome to aid the identification of genes that underlie susceptibility to common complex disease by association studies (<http://www.hapmap.org>). From the late 1980s, LD had been used successfully to fine-scale map a number of comparatively rare Mendelian disorders in which high-risk alleles and founder effects predominate (Kerem et al. 1989; MacDonald et al. 1991; Lerner et al. 1994; Sirugo et al. 1994; Puffenberger et al. 1994; Brzustowicz et al. 1995; Goddard et al. 1996). By the mid-1990s, it was speculated that modest-risk alleles associated with common disease such as type 2 diabetes or schizophrenia might be revealed by whole-genome comparisons between affected and control populations (Risch and Merikangas 1996; Lander 1996).

From the outset, it was recognised that the direct approach of cataloguing all common variants in coding and regulatory regions of genes would be a massive task, and that an indirect approach of relying on LD between the risk locus and nearby polymorphisms might be more efficient. Early simulations, based unrealistically on randomly distributed crossovers and constant population growth, painted a pessimistic picture with levels of LD unlikely to extend beyond 3 kb in the general population and implying that associa-

tion studies would require very dense SNP maps (Kruglyak 1999). However, empirical data from a handful of regions led to the appreciation that LD can extend for tens of kilobases or more and is probably not infrequent in the human genome (Dunning et al. 2000; Abecasis et al. 2001; Daly et al. 2001; Johnson et al. 2001; Patil et al. 2001; Reich et al. 2001; Dawson et al. 2002). Indeed, sperm studies in the MHC suggested that clustering of crossovers into hotspots is largely responsible for creating extended LD blocks (Jeffreys et al. 2001), although blocks can also arise by chance through random historical crossover events that have fortuitously spread via drift (Subrahmanyam et al. 2001; Zhang et al. 2002; Wang et al. 2002; Phillips et al. 2003). Arguably, the precise reason for LD blocks may be unimportant from a gene hunting perspective. However, it is unquestionable that this structuring of diversity into LD blocks can greatly simplify whole-genome association studies by using an efficient subset of non-redundant SNP markers to capture the information content of haplotypes, so-called haplotype tagSNPs (htSNPs) or tagSNPs (Johnson et al. 2001). This strategy would, however, miss any aetiological variants located within recombination hotspots.

The HapMap project's primary aim is to establish the haplotype structure of the entire human genome to facilitate tagSNP selection. In total, 269 individuals are being examined from four geographical populations (usually considered as three analysis panels), namely the Yoruba people of the Ibadan region in Nigeria, the Japanese of Tokyo plus the Han Chinese of Beijing, and Utah residents with ancestry from northern and western Europe. Phase I of the project was completed in October 2005 yielding data on more than one million validated SNPs with an average density of 1 per 5 kb, though centromeres, telomeres and segmental duplications were inevitably under-represented and the non-recombining mitochondrial DNA and Y chromosome were each treated as exceptions (Altshuler et al. 2005). Markers showing non-Mendelian inheritance, deviations from Hardy-Weinberg equilibrium and genotyping inconsistencies have been excluded (though in fact these can give valuable clues about structural polymorphisms in the human genome (Conrad et al. 2006)). Emphasis has been placed on markers with minor allele frequencies of 5% or greater, since these conform to the common disease/common variant hypothesis (Reich and Lander 2001) and should ultimately be more valuable in terms of diagnostics and possible intervention (Hinds et al. 2005). The Yoruban and Utah analysis sets each comprise 30 parent-offspring trios, allowing tests of the accuracy of a variety of algorithms for phasing diplotypes (Marchini et al. 2006); the most accurate, PHASE (Stephens and Donnelly 2003), has been applied across the whole data set. To directly evaluate the effect of SNP density on data interpretation, HapMap has been complemented by the ENCODE project ([ENCyclopedia Of DNA Elements](#); The ENCODE Project Consortium 2004). Five hundred kilobases from each of ten regions, which in aggregate approximate the genome-wide average for GC content, gene density, pedigree-based recombination rate

and percentage of sequence conserved relative to the mouse, have been re-sequenced from each of 48 chromosomes across the three analysis panels to capture almost all the common SNPs in each region.

A similar effort to establish whole genome patterns of common DNA variation has been made by Perlegen Sciences Inc. (Hinds et al. 2005). This complementary data set consists of 1.6 million high-quality genotyped SNPs in 71 unrelated individuals from three populations (24 European Americans, 23 African Americans and 24 Han Chinese from the Los Angeles area). Two-thirds of the sequenced genome is covered by inter-SNP intervals of less than 10 kb, with on average 1871 bp between adjacent SNPs. Nine of the European Americans are also part of the HapMap project and for almost 157 000 SNPs there is > 99.5% concordance of genotypes between the studies.

Data emerging from these large-scale projects confirm the findings of previous studies restricted to small parts of the genome; long regions of LD are ubiquitous and generally show limited haplotype diversity and extensive SNP redundancy (Altshuler et al. 2005). These effects are more pronounced for non-Africans, consistent with a recognised history of recent population bottlenecks and reduced DNA diversity (Hinds et al. 2005). Although nearly 50% of markers identified by the ENCODE re-sequencing project were rare, 90% of heterozygous sites in each individual were due to common variants. Each of these was perfectly correlated with between three and ten others, and partially correlated with many others. There is still much debate as to how to exploit this redundancy most effectively for association studies (Schwartz et al. 2003; Phillips et al. 2003; Ke et al. 2004; Sun et al. 2004; Nothnagel and Rohde 2005), to what extent tagSNP sets will prove to be shared by different populations (González-Neira et al. 2006), and how effectively common SNPs can be used to tag rarer SNPs (Ahmadi et al. 2005; Lin et al. 2004; Kamatani et al. 2004; Zeggini et al. 2005; Clark et al. 2005; Wiltshire et al. 2006). These are issues beyond the scope of this review.

In July 2006, HapMap released the complete phase II data set containing an additional ~ 4.6 million SNPs. This has increased coverage to on average one marker per kilobase, and will provide a highly detailed picture of LD within the human genome. At the time of writing, no formal descriptions or detailed analyses of this data set have been published.

3.1

Genome-Wide Patterns of Recombination

HapMap and allied projects have also provided population geneticists with an invaluable resource from which genome-wide patterns of recombination may be inferred at varying levels of resolution. This has been embraced with great enthusiasm as it raises the possibility of identifying specific genomic features that correlate with, and potentially control, recombination rate (McVean et al. 2005).

Model-free methods that just detect recombination events, for example the conceptually simple “four gamete test” which assumes infinite site mutation and infers a recombination event between two markers if all four gametes are observed in the sample (Hudson and Kaplan 1985), greatly underestimate true levels of historical recombination and fail to appreciate the inherent stochastic nature of population data (Stumpf and McVean 2003). For these reasons, much attention has been given to modelling the underlying evolutionary process using the coalescent² (Nordborg 2001). Since it is very difficult to disentangle the effects of recombination from mutation, selection, drift and demography (McVean et al. 2004), these approaches attempt to reconstruct the genealogy of haplotypes and derive the population recombination rate ρ , which is equivalent to $4N_e r$, where N_e is the effective population size and r is the per generation recombination rate as measured by pedigree or sperm-typing approaches. N_e is influenced by the demographic history of the particular population studied, and may therefore differ between populations (Stumpf and McVean 2003). Full-likelihood approaches attempt to use all data available but are computationally very intensive (Griffiths and Marjoram 1996; Kuhner et al. 2000; Nielsen 2000; Fearnhead and Donnelly 2001). To overcome this, other methods that just use summary statistics from coalescent modelling have been developed but the data derived from these can be compromised (see Wall 2000). Instead, composite-likelihood approaches have been more generally accepted, wherein subsets of the data are analysed separately and their likelihoods combined (Hudson 2001; Fearnhead and Donnelly 2002; Li and Stephens 2003).

The most widely used method is LDhat (McVean et al. 2002), which fits a statistical model based on the coalescent to patterns of LD and then estimates ρ within a Bayesian framework in which the prior distribution favours short-range uniformity in recombination rate. The Hotspotter algorithm of Li and Stephens (2003) also directly relates LD patterns to the underlying recombination rate, but considers all loci simultaneously and explicitly allows for variation in recombination rate. Both approaches have been validated by extensive simulation and by comparison to pedigree and sperm data sets, and have been applied to relatively small intervals of the genome (McVean et al. 2004, Crawford et al. 2004; Tiemann-Boege et al. 2006). For LDhat this has recently been extended to the complete Phase I HapMap data set (Myers et al. 2005).

² The patterns of variation seen between contemporary haplotypes is dependent on the shape of the tree that depicts their ancestry and this in turn is a consequence of the specific evolutionary processes and demographic influences encountered. The coalescent represents the state wherein these extant haplotypes have been traced *backwards* in time to their most recent common ancestor. Coalescent theory focuses on these genealogical descriptions, the mathematical modelling of which is simpler and more efficient than considering the evolution of such variation in populations *forwards* in time since only *sampled* haplotypes need be considered. Under specified models, plausible genealogies can be simulated and parameters, such as the underlying population recombination rate, can be inferred.

Information on population recombination rates can also be gleaned from LDU maps that have additive distances, much like linkage maps, and locations which can be related to both genetic and physical maps (Maniatis et al. 2002). LDU maps are based on the Malecot model of decay of association with distance governed by recombination rate (Malecot 1948) and were developed to describe the extent of LD useful for association mapping. The maps consist of a series of plateaus corresponding to LD blocks separated by steps marking putative recombination hotspots. Overall, the broad contours of chromosome-specific maps show close correspondence to the high-resolution linkage map (De La Vega et al. 2005) and at a fine level, major inflexions have been shown to coincide with recombination hotspots characterised by sperm analyses (Zhang et al. 2002). An LDU map has been constructed for the entire genome based on the phase I HapMap data (Tapper et al. 2005). Despite the very different theoretical basis (see Tapper et al. 2005 for discussion), the global picture of recombination emerging from this approach appears very similar to that inferred from coalescent analyses.

At the megabase scale, genome-wide historical recombination profiles inferred from population data show good correspondence to contemporary rates estimated from pedigree analyses. By and large, positive correlations of recombination rates with GC content, imprinted chromosomal regions and nucleotide diversity are noted, observations of higher rates of recombination in telomeric regions are upheld and patterns are largely conserved across populations despite differences in population history and SNP spacing (McVean et al. 2004; De La Vega et al. 2005; Hinds et al. 2005; Myers et al. 2005; Serre et al. 2005; Sandovici et al. 2006). These findings are consistent with general inferences from the comparative linkage maps of the rat, mouse and human (Jensen-Seaman et al. 2004). At this scale, human recombination rates fluctuate over large distances with regions of low recombination tending to be more extensive than regions of high rate (McVean et al. 2004). However, apart from centromeric regions, these types of analyses show no evidence for true recombination deserts as described by Yu et al. (2001), and indeed no single autosomal interval greater than 0.2 Mb has been totally devoid of historical recombination (Myers et al. 2005).

When viewed at the kilobase level, recombination rates derived from the HapMap project reveal considerably more variation than is captured by pedigree analysis. As predicted by sperm crossover analyses, the fine-scale genome-wide recombination landscape is dominated by sharp peaks in rate that most likely equate to recombination hotspots, with about 80% of all recombination events occurring within at most one fifth of the genome sequence (Myers et al. 2005). When analysed at fine resolution, it is apparent that the broad regions of relatively increased recombination rate result from an increased density and/or intensity of these very narrow hotspots.

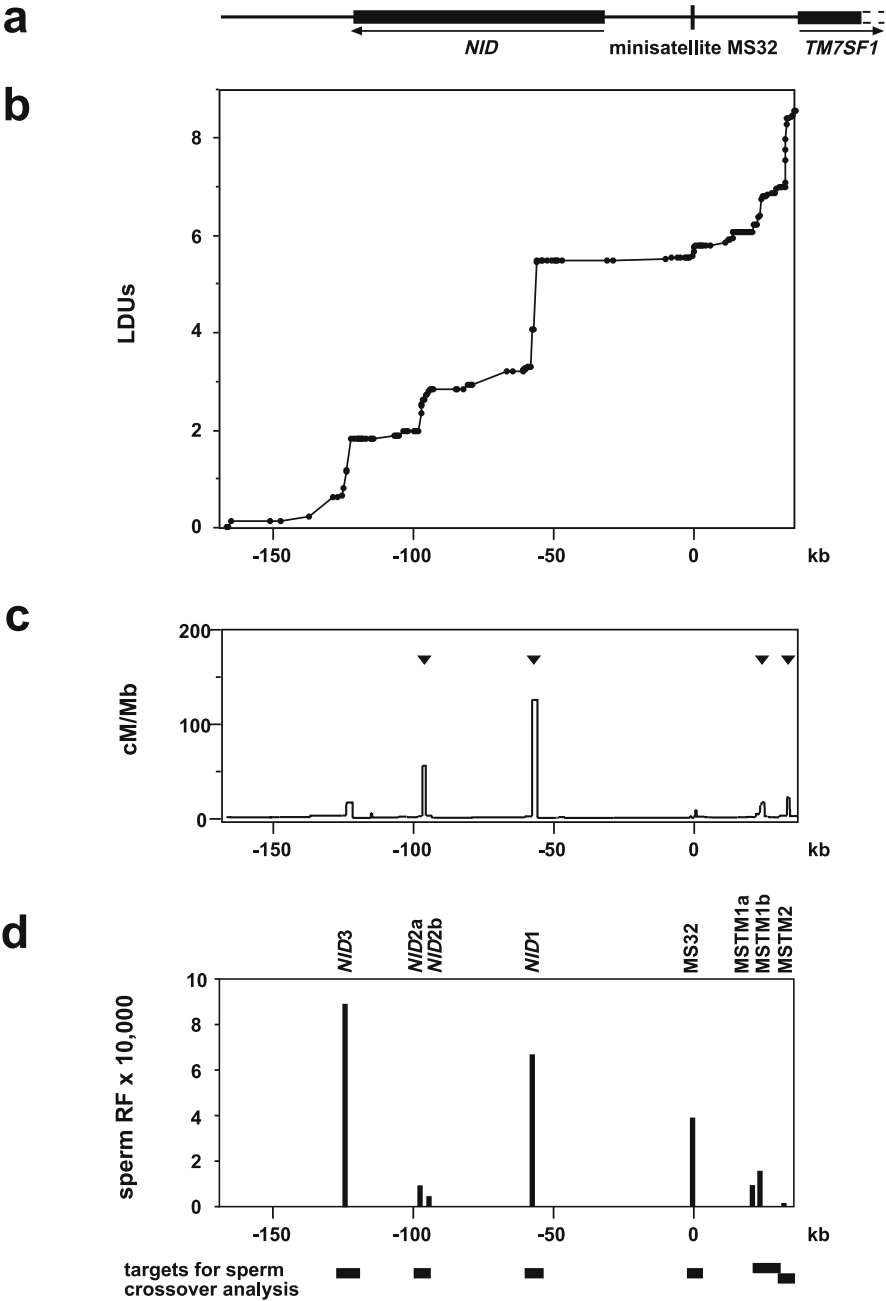
3.2

Detecting Hotspots from Population Data

Several of the approaches have the ability to identify putative recombination hotspots from population data with considerable precision (Fig. 3). With the Hotspotter program (Li and Stephens 2003), each haplotype is reconstructed as a mosaic of previously considered haplotypes, allowing the local background recombination rate to be estimated from the average length of mosaic pieces, and the location and intensity of hotspots to be estimated from the positions of breaks within the mosaic. Significance is assessed by a Bayes factor that takes into account both chance clustering of events and the probability of recombinant haplotypes drifting to high frequency. This method has most notably been used by the SeattleSNPs program to examine the fine-scale patterns of recombination over 74 genes implicated in lung, cardiovascular and inflammatory disease (Crawford et al. 2004).

LDhot, conceptually akin to LDhat, compares the likelihood of a model with randomly distributed crossovers with one in which the central 2 kb of a chosen interval is a recombination hotspot and assesses significance with a suitably matched null data set generated by simulation (McVean et al. 2004; Winckler et al. 2005). This has been applied to the three HapMap populations and a hotspot declared if at least two show evidence at the 5% significance level and the other at the 1% level. Hotspots are centred in the interval that gives the maximum rate and which is at least twofold greater than adjacent 2-kb windows. In practice this tends to equate to intervals of at least 5 kb resulting in artefactually broadened hotspots compared with those characterised by sperm typing.

Fig. 3 Patterns of historical and contemporary recombination in a 206-kb interval on chromosome 1q42.3. **a** The region was known to contain a sperm crossover hotspot located next to the highly variable minisatellite MS32 (*D1S8*) (Jeffreys et al. 1998b) in a 77-kb non-coding region between the *NID* and *TM7SF1* genes. **b** The LDU map of the region derived from SNP genotype data from 80 sperm donors. *Plateaus* correspond to LD blocks and *steps* mark putative crossover hotspots. **c** Historical recombination rates estimated from coalescent analyses of the same high-density SNP genotype data using the LDhot method. *Triangles* mark putative hotspots significant at $P < 0.01$. Note that the locations of these hotspots show good correspondence to those identified by the LDU map, although the step sizes of the latter do not perfectly reflect the historical recombination frequency owing to the different theoretical basis of this analysis. **d** Sperm crossover frequencies. Six intervals identified as containing putative hotspots were chosen for crossover analysis in two to seven men per target. In total, eight hotspots were identified, including unexpectedly some in regions of strong marker association. Historical activity inferred from population genetic analysis correlates poorly with contemporary male frequencies. For example, hotspot *NID3* was predicted to be weak from the coalescent analysis but in fact proved the most intense in sperm. Data taken from Jeffreys et al. (2005) and unpublished results



Phase I HapMap data revealed in excess of 25 000 candidate hotspots in the human genome with an average density of around 1 per 50–100 kb (Myers et al. 2005). By its nature, the HapMap project gives a sex-averaged view of historical recombination. However, attempts have been made to tease out sex-specific information by focussing on regions of the deCODE linkage map that differ in recombination proficiency between the sexes, as well as by examining the X chromosome which can only recombine at female meiosis. These analyses have for the first time inferred the widespread existence of female hotspots (directly accessing the human female germline is not feasible), but have failed to demonstrate any clear difference in overall distribution or intensity of hotspots between the sexes that might explain the difference in linkage map lengths. Although the total number of hotspots approximately mirrors the predicted number of genes in the genome, human hotspots do not conform to the α hotspot model described in yeast, wherein hotspots associate with sites bound by transcription factors and preferentially map to the 5' ends of genes (Petes 2001). Instead, most candidate hotspots are within 50 kb of a gene but are preferentially located outside the transcribed domain and are typically found around ± 30 kb from the start codon.

To look for fine-scale genomic features that might be predictors of recombination proficiency, a complementary data set of cold spots was generated, matched in number, size and SNP density to candidate hotspots (Myers et al. 2005). The long terminal repeats of the THE1A and THE1B retrovirus-like transposons, CT-rich and GA-rich repeats were over-represented in hotspots, whilst (TA)_n repeats, GC-rich repeats and certain L1 elements were significantly under-represented. The strongest correlation of all was seen with a seven nucleotide long motif (CCTCCCT) often associated with THE1 elements. Outside the context of THE1A/B, the CCTCCCT motif appears to confer a smaller probability of hotspot activity but is far more frequent in the genome. At most, this motif accounts for 11% of the 25 000 hotspots examined. In addition, a nine nucleotide long motif (CCCCACCCC) is enriched amongst HapMap hotspots. CCTCCCT and CCCCCACCCC motifs exist at the centre of hotspots *DNA2* and *NID1*, respectively; these hotspots have been defined by sperm typing and crossover rate polymorphism data (Jeffreys and Neumann 2002, 2005) and provide strong evidence that these motifs may directly influence hotspot activity (Myers et al. 2005) (Sect. 4.1). It will be of great interest to see whether full analysis of the phase II HapMap data will lead to better definition of candidate hotspots and reveal sequence motifs with greater explanatory power for recombination hotspots.

4

Current Picture of Allelic Recombination

4.1

Evolution of Hotspots

Recent comparisons of DNA diversity in humans and chimpanzees have revealed remarkably divergent LD landscapes at orthologous loci despite $\sim 99\%$ identity at the DNA sequence level (Wall et al. 2003; Ptak et al. 2004; Ptak et al. 2005; Winckler et al. 2005). This suggests that major turnover of hotspots has occurred within the last six million years to an extent disproportionate to sequence divergence, and raises major questions about the control of hotspot activity by local DNA sequence determinants. Rapid hotspot turnover also raises the possibility of hotspot polymorphism within extant human populations arising from newly evolved hotspots and from hotspots on their way to extinction. Population data generated for European Americans and African Americans by the SeattleSNPs Program suggest that the intensity of some hotspots may differ between populations (Crawford et al. 2004). Similar inferences have been made for a 10-Mb stretch of chromosome 20 examined in four population samples (Evans and Cardon 2005). However, these population approaches seem to be rather poor predictors of current recombination rates, with several instances of very substantial differences between historical rates and sperm crossover frequencies (Fig. 3). The most extreme examples include a region of extreme LD breakdown that is in fact recombinationally inert in sperm (Kauppi et al. 2005), and examples of hotspots “hidden” in regions of strong marker association (Jeffreys et al. 2005). The former might represent a female-specific hotspot or a hotspot on its way to extinction, while the latter are candidates for young hotspots that have failed to leave a significant mark on haplotype diversity. By their very nature, population-based analyses cannot detect the polymorphism in activity between individuals predicted from rapid hotspot turnover.

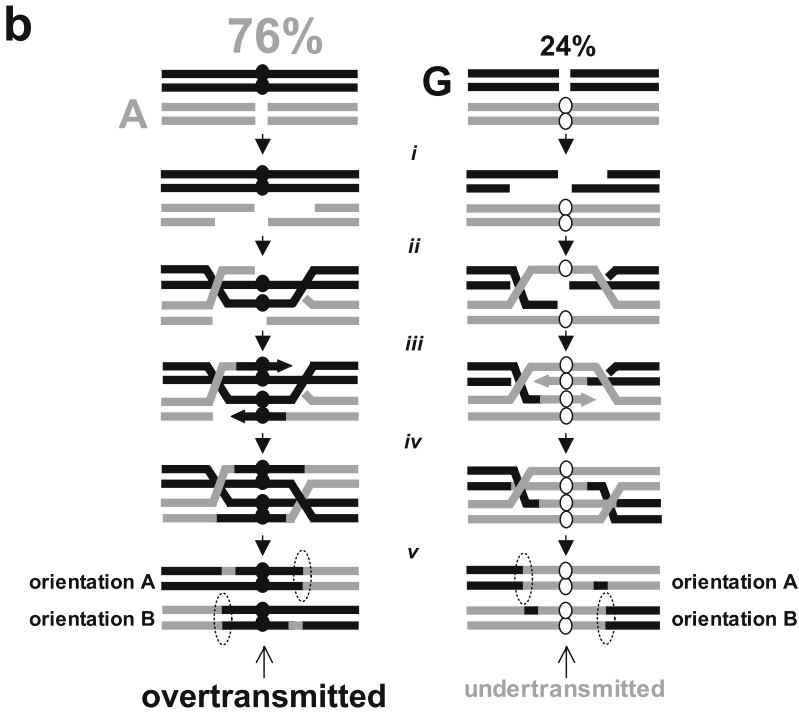
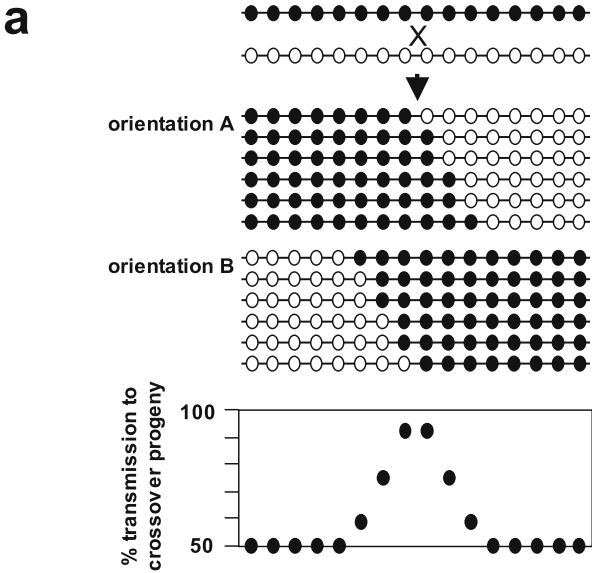
In contrast, sperm typing can directly address the issue of crossover rate variation between men and can provide greater insight into the issue of hotspot evolution. Such polymorphism can be detected by two approaches. The first is simple crossover frequency variation detected in sperm; single DNA molecule analysis is quantitative but cannot readily be used to detect subtle ($< \text{twofold}$) differences between men in recombination activity. The second approach is the reciprocal crossover asymmetry test (Jeffreys and Neumann 2002). This is a powerful rate-independent test that examines the distribution of exchange points to test whether reciprocal exchanges are distributed differently across a hotspot and can be used to detect even minor variation in crossover activity. To date, these tests have revealed polymorphism in activity in at least eight of 19 hotspots analysed, demonstrating that this is a common feature of human hotspots (Jeffreys and Neumann 2002,

Fig. 4 Reciprocal crossover asymmetry and meiotic drive. **a** The asymmetry test. Asymmetry arises if reciprocal exchange points in orientation A crossovers map to locations different from those in orientation B, as shown. With asymmetry, markers closest to the centre of the hotspot show the strongest non-Mendelian transmission into crossover progeny. **b** A model for biased gene conversion leading to asymmetry at the *DNA2* hotspot. Recombination is initiated by a DSB at or very close to an A/G SNP, but occurs more frequently on the haplotype bearing the A allele (grey) than the G allele (black). Resection produces 3' overhangs (i) that invade the other chromosome (ii). Mismatches are removed from the invading strands and the gap repaired with information from the non-initiating chromosome (iii). Isomerization (iv) and resolution of Holliday junctions (v) yield reciprocal crossover products containing only the non-initiating allele, along with any other markers from the same haplotype that happen to fall within the resection/repair tract. Exchange points for orientation A and B crossovers thus map to different intervals (dashed lines). The imbalance in initiation leads to the suppressing G allele being over-transmitted to crossover progeny. Data taken from Jeffreys and Neumann (2002)

2005; Jeffreys et al. 2005; Neumann and Jeffreys 2006; Tiemann-Boege et al. 2006).

The reciprocal crossover asymmetry test also allows recombination proficiency at the level of individual haplotypes to be analysed (Jeffreys and Neumann 2002) (Fig. 4a). For many hotspots, exchange points always map to the same intervals regardless of the orientation in which the recombinants are selected. However some hotspots show reciprocal crossovers in some men that map to different locations. This asymmetry has been well documented at the *DNA2* hotspot in the MHC and at the nearby *NID1* and *MSTM1a* hotspots on chromosome 1q42.3, and may well occur at the MS32 hotspot located between *NID1* and *MSTM1a* (Jeffreys and Neumann 2002, 2005; Neumann and Jeffreys 2006). In all cases of men showing asymmetry, the reciprocal crossover distributions are displaced by ~ 400 bp and are accompanied by over-transmission of hotspot alleles from one haplotype into recombinant progeny. This biased gene conversion accompanying crossover arises from repair of a DSB on the initiating haplotype using information from the other haplotype, and allows relatively active and suppressed haplotypes to be identified in men carrying chromosomes with different recombination initiating activities.

For hotspots *DNA2* and *NID1*, the cause of asymmetry can be traced to heterozygosity at a single SNP located closest to the hotspot centre. A single base variation within a hotspot can therefore directly influence initiation rates and thus cause polymorphism in crossover activity. For hotspot *DNA2*, the SNP is an A/G polymorphism located ~ 5 bp from the centre, with the suppressing G variant being over-transmitted to about 75% of crossover progeny in A/G heterozygotes. AA and GG homozygotes show no asymmetry, and GG homozygotes show, as expected, rather lower crossover rates than AA men. Significantly, the G variant disrupts the CCTCCT hotspot motif (Myers et al. 2005). A remarkably similar picture is seen at hotspot *NID1*, with a cen-



tral C/T polymorphism ~ 70 bp from the hotspot centre causing asymmetry through suppression of initiation by the T allele which disrupts the CCCCAC-CCC motif (Myers et al. 2005). These data provide the best evidence to date that human hotspot activity can be influenced by primary DNA sequence determinants within a hotspot. However, these cases do not represent instances of presence/absence polymorphism of hotspots; rather, the suppressing SNP alleles only reduce initiation activity by a modest factor of $\sim$ fivefold and even homozygous suppressed men show crossover activity within the hotspot.

These transmission biases in favour of recombination suppressing alleles result in subtle but significant departures from a 50 : 50 Mendelian segregation ratio at the population level. For example at *DNA2*, since the majority of gametes are non-recombinant, the ratio is estimated at 50.00049 : 49.99951, while the degree of population distortion at *NID1* is 18-fold more intense due to the higher crossover rate (50.009 : 49.991) (Jeffreys and Neumann 2002, 2005). These systematic biases associated with crossover asymmetry constitute a weak form of meiotic drive (Zimmering et al. 1970), in which any new alleles that suppress recombination, together with closely linked markers, are favoured for fixation. At *NID1*, the strength of drive is sufficient to virtually guarantee that the suppressor will eventually sweep to fixation. This deterministic drive should cause active hotspots to be doomed to extinction, or at least to attenuation of activity, giving rise to the so-called hotspot paradox, namely how active hotspots can arise and persist in a population (Boulton et al. 1997, Pineda-Krch and Redfield 2005). However, meiotic drive is unlikely to eliminate a hotspot completely; as activity declines, so will the strength of drive, until the population frequency of any suppressor alleles will be determined by drift alone, irrespective of their ability to block initiation.

The flip side to the coin is how hotspots arise in the first place. The observation of local competition between hotspots in yeast has led to the suggestion that as one human hotspot dies out the relative rates of others in the vicinity may increase (Pineda-Krch and Redfield 2005; Myers et al. 2005). This model would leave overall recombination rates conserved over large scales, but has yet to be systematically tested using sperm approaches. Recent data on hotspots PC4-1a and PC4-1b some 3 kb apart on chromosome 21 have been interpreted as representing a transition stage in human hotspot evolution consistent with this theory (Tiemann-Boege et al. 2006). Of the three men analysed, two are active at PC4-1a and repressed at PC4-1b, whilst the third is active at PC4-1b and repressed at PC4-1a. Only a modest difference in rate is observed across the entire interval for these individuals, compatible with a concerted change in recombination profile.

In contrast, MSTM1a and MSTM1b are two hotspots that appear to be entirely independently regulated despite being just 2 kb apart (Neumann and Jeffreys 2006). Hotspot MSTM1b is active in all men tested to date but shows a remarkable 75-fold range in crossover frequency between men. In contrast, MSTM1a is active in only 10% of men and totally inactive in all others

tested. This is the first clear example of a hotspot showing presence/absence polymorphism in humans. There is no significant correlation in activity at these two hotspots—for instance, men can be found with similar activities at MSTM1b but with MSTM1a either on or off—arguing against a simple model of balanced co-regulation of nearby hotspots.

Remarkably, haplotype and DNA sequence analysis across the MSTM1 hotspots has shown that presence/absence polymorphism at MSTM1a, as well as activity variation at MSTM1b, does not correlate whatsoever with any local DNA sequence variation either within the hotspots or kilobases either side (Neumann and Jeffreys 2006). For example, pairs of men sharing precisely the same haplotypes for at least 100 kb around MSTM1 can show MSTM1a either active or suppressed. This provides clear evidence that hotspot activity is not specified solely by local DNA sequence determinants. Given precedence for distantly located *cis*-acting regulators of mouse hotspots (Shiroishi et al. 1991) and clear evidence of the same in *S. pombe* (Cervantes et al. 2000; Young et al. 2002), it is tempting to speculate their existence here. However, if this is the case, then a number of intervening hotspots known to exist in the MSTM region would have to be unaffected by these remote elements.

Hotspot MSTM1a not only shows presence/absence polymorphism but is also a candidate young hotspot, being embedded within a region of extreme marker association with no evidence of obligate historical recombination (Jeffreys et al. 2005). Just how the hotspot became activated in the absence of a local DNA sequence change remains unclear. One possibility is epigenetic activation through histone modification or DNA methylation that might vary more substantially than DNA sequence. Associated changes in local chromatin conformation might allow initiation of recombination and in support of this, imprinted regions of the genome can show differential recombination patterns, certainly over broad scales (Paldi et al. 1995; Robinson and Lalande 1995). But quite how an epigenetic signal like DNA methylation could survive germline re-programming to maintain an influence on hotspot activity remains a mystery. Instead, it seems more plausible that the signal—and therefore its influence—may be transient, a so-called epimutation (Suter et al. 2004). If true, then hotspots may actually appear and disappear on even faster timescales than previously imagined, from one generation to the next.

4.2

Mechanistic Insights

Although sperm studies have covered just 0.03% (0.8 Mb) of the genome, they have provided us with an unprecedented view of processes involved in human recombination. The constant width of all characterised hotspots points to a common underlying mechanism, whilst symmetric crossover distributions indicate a bidirectional distribution of crossover resolution points centred on an initiation site. This is totally consistent with the double-strand break re-

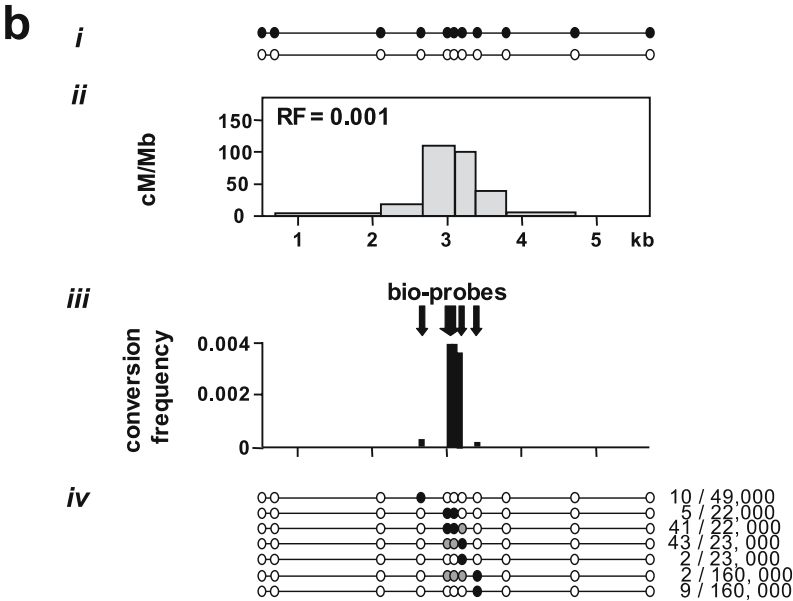
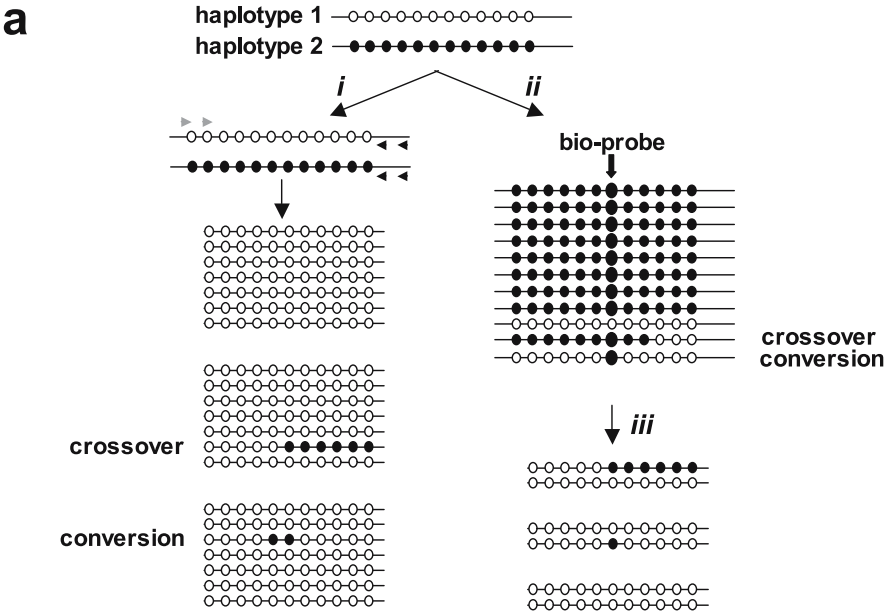
Fig. 5 Detecting gene conversion events in sperm. **a** Methods of isolating recombinants from men with multiple SNP heterozygosities. (i) The “half-crossover assay”. Small pools of sperm DNA are amplified by nested PCR using allele-specific primers directed to haplotype 1 (*grey arrows*) plus universal (not allele-specific) primers (*black arrows*) to amplify haplotype 1 molecules. Crossover and conversion molecules in these pools are detected by the presence of markers from haplotype 2. (ii) Hybridisation enrichment of sperm DNA using a biotinylated allele-specific probe (bio-probe) directed to a single SNP site largely removes most haplotype 1 molecules. (iii) Enriched DNA is then screened as in (i). Enrichment allows many more molecules to be screened per pool and also validates recombinants, all of which must show a switch at the selected site. **b** Distribution of crossovers and conversions at the MHC hotspot *DNA3*. (i) Parental haplotypes of an informative man. (ii) Sperm crossover activity across the hotspot. The recombination activity of each interval was estimated from the sperm crossover frequency and the physical distance between markers. The recombination fraction (RF) over the entire interval is shown. (iii) Frequency of conversions detected following enrichment with bio-probes as arrowed. (iv) Structures of conversions. Selected SNP sites are shown in *black* and unselected co-converted sites in *grey*. Numbers of convertants and the numbers of progenitor DNA molecules screened are given on the right. Data taken from Jeffreys and May (2004)

pair model (DSBR) proposed for yeast meiotic recombination (Szostak et al. 1983; Sun et al. 1991); recombination is initiated by a DSB followed by 5′–3′ resection to give 3′ single-strand overhangs, one of which invades the other homologue and functions as primer for DNA repair synthesis³.

This model can accommodate the four orders of magnitude difference in recombination frequency between human hotspots as a consequence of differences in the efficiency of recombination initiation. Indeed, the most satisfactory explanation of crossover asymmetry invokes just such a difference but at the level of individual haplotypes; DSBs occur more frequently on haplotypes carrying the non-suppressor allele, and following resection and strand invasion, this initiating chromosome is repaired with information from the opposite haplotype carrying the suppressor allele (see Fig. 4b). Asymmetry therefore represents a form of biased gene conversion accompanying crossover and as predicted by this model is most strongly observed for markers closest to the initiating lesion.

Detection of de novo non-exchange gene conversion events that are not accompanied by crossover is technically challenging. However, sperm typing methods have been developed that allow these events to be detected and quantified, and have provided further insights into the mechanisms of human meiotic recombination. In the “half-crossover assay”, allele-specific primers upstream of a hotspot are used to amplify just one haplotype from small pools of sperm DNA, and the resulting products are then typed with allele-specific oligonucleotide probes to detect markers from the other haplotype that would signal the presence of conversions as well as crossovers. The sec-

³ For alternative models to Szostak’s assumption of two Holliday junctions, see chapters of Smith and Haber.



ond approach uses DNA enrichment by allele-specific hybridization prior to PCR (Jeffreys and May 2003) to improve the signal-to-noise ratio and scale up the screening process, allowing the detection and validation of even rare conversion events (frequency 10^{-6} or lower), including those that affect just a single marker (Jeffreys and May 2004; Fig. 5).

Most hotspots investigated to date are active in gene conversion as well as crossover, with the centre of a crossover hotspot being most active in gene conversion. This indicates that conversions and crossovers most likely derive from the same recombination-initiating lesions (Jeffreys and May 2004; Jeffreys and Neumann 2005). This is strongly supported by the finding that true gene conversion events at the *NID1* hotspot exhibit exactly the same degree of bias that results in crossover asymmetry, with the suppressor allele being over-transmitted to the more active haplotype in both crossover and non-exchange conversion progeny (Jeffreys and Neumann 2005). In all cases, conversion tracts are very short, with a mean length indeterminate but of the order of 60–300 bp; as a result, most events involve the transfer of just a single SNP between haplotypes, generating a very steep gradient of activity extending in both directions from the hotspot centre. However, the latter cannot represent a unique DSB site since not all conversions share a common region of overlap; instead, recombination-initiating lesions must be diffused over a very localised zone probably of the order of 400–500 bp wide (Jeffreys and May 2004), similar to the distribution of meiotic DSBs at yeast hotspots (Keeney 2001). Crossover hotspots thus contain a narrower gene conversion hotspot.

The observed relative rates of conversion to crossover show major variation between hotspots, ranging from $\sim 4 : 1$ to $< 1 : 12$. The ability to detect conversion events is influenced by marker density at the centre of the hotspot, but this alone cannot account for the disparity between loci. Thus, an 11-fold discrepancy is noted between *NID1* and *DNA3*, even though the most informative markers are similarly placed from their hotspot centres at 70 and 85 bp, respectively (Jeffreys and Neumann 2005), and no conversions have yet been detected at the β -globin hotspot despite a centrally located informative marker (Holloway et al. 2006). We currently have no clues about factors that influence the balance between crossovers and conversions in humans, nor whether they are products of DSB processing down entirely separate pathways as seen in some model organisms (Paques and Haber 1999; Guillon et al. 2005).

4.3

Lessons from Mice

Two mouse hotspots, *E β* and *Psmb9*, initially detected by pedigree analyses (Guillon and de Massy 2002; Yauk et al. 2003; Guillon et al. 2005), have been characterised using gametic DNA approaches with precision comparable to

human hotspots. They show remarkable similarity to their human counterparts in terms of crossover distribution, asymmetry effects and patterns of gene conversion. The *Psmb9* hotspot exhibits an exceedingly high frequency of recombination ($> 1\%$), making it a particularly tractable locus for study, and is equally active in the female as the male germline. In a *Spo11*^{-/-} background, there is a dramatic reduction in the frequency of both crossovers and conversions in oocytes indicating that as in yeast (Keeney et al. 1997), the meiosis-specific Spo11 protein catalyses DSB formation, and that the resulting lesions can give rise to both types of recombinant molecule (Guillon et al. 2005). In the absence of the mismatch repair gene *Mlh1*, a 9- to 20-fold reduction in frequency of crossovers is seen in both oocytes and sperm with a clear change in the spectrum of events (Guillon et al. 2005). Although more than 85% of these crossovers are simple as in wild-type mice, exchange points are more tightly clustered suggesting alternative processing. The remaining events have complex and hitherto unprecedented structures either with exchange points outside the previously mapped hotspot, or multiple exchange points indicating patchy gene conversion, or significant stretches of heteroduplex DNA. Clearly, the major crossover pathway is *Mlh1*-dependent, being implicated in greater than 90% of exchanges (cf. just 30–50% for yeast; Guillon et al. 2005), but it remains to be seen what other pathways are involved. Finally, it is noteworthy that the frequency and nature of gene conversion events is totally unaltered in *Mlh1*^{-/-} mice, providing the first direct evidence that mammalian crossovers and conversions can result from distinct DSBR pathways just as in yeast (Paques and Haber 1999; Guillon et al. 2005).

Unquestionably, mouse models will continue to reveal the molecular machinery involved in mammalian meiotic recombination, and indeed considerable progress has already been made (see Brugmans et al. 2006). A complementary approach is to analyse the DNA intermediates of recombination directly in meiotic cells in a manner similar to the characterization of recombinants from sperm. The latter gives direct information only on recombination resolution points but not on recombination initiation or downstream processing. A sensitive method has been developed to detect meiotic DNA breaks in mouse testis DNA and this has been used to establish the temporal appearance of site-specific breaks (Qin et al. 2004). Another approach to analysing recombination initiation may develop from the recent discovery in yeast and mice of short oligonucleotides attached to Spo11p that appear to be derived from recombination-initiating DSBs (Neale et al. 2005). It remains to be seen if similar approaches can be used to quantify and precisely map initiation sites across the entire human genome, or indeed if other methods can be developed to characterise resection tracts or establish the distribution of predicted double Holliday junction intermediates. Though extremely challenging, these lines of investigation hold great promise in providing new mechanistic insights into this fundamental process.

4.4

The Relationship Between Recombination and Sequence Diversity

The positive correlation between recombination and sequence diversity noted for humans (Nachman et al. 2001) is also mirrored by correlations between recombination and DNA divergence in human/mouse comparisons (Lercher and Hurst 2002) and between human and chimpanzee (Hellmann et al. 2003; Bussell et al. 2006). Many explanations have been put forward to explain these relationships (see Hurles 2005 for discussion), but two relate directly to the process of recombination. The first is that recombination itself is directly mutagenic through errors in DNA repair synthesis (Lercher and Hurst 2002). This has yet to be directly tested experimentally on crossover progeny. The second is that recombination and mutation are positively correlated with each other because they are each correlated with GC content (Fullerton et al. 2001). Diversity studies have suggested that GC-biased gene conversion could provide the link between recombination and GC content (Eyre-Walker et al. 1993; Marais 2003; Webster and Smith 2004), whilst the enhanced mutability of the CpG doublet could account for the elevated mutation rate in regions of high GC content (Nachman and Crowell 2000). Indeed, recent analysis of the HapMap data points to a weak but significant GC fixation bias that is strongest at the centre of inferred recombination hotspots (Spencer 2006). However, the limited sperm studies carried out to date have yet to capture this process in action.

5

Ectopic Recombination

Unequal crossover or ectopic recombination has long been recognised as of fundamental importance in the evolution of gene families (Ohno 1970) and as a means of maintaining locus diversification (e.g. Kelly et al. 2005), and has more recently been implicated in so-called genomic disorders (Lupski 1998). Initiated by misalignment of non-allelic but highly homologous DNA sequences, and hence also referred to as non-allelic homologous recombination (NAHR), this form of exchange is predicted to result in intra-molecular, intra- or inter-chromosomal deletions and duplications, inversions or translocations depending on the location and relative orientation of the mediating repeat sequences (Fig. 6a).

Large-scale rearrangements of this nature have been recognised cytogenetically since the late 1950s, but only since the completion of the Human Genome Project has the duplicated nature of our genome—and hence the potential for NAHR—been fully appreciated. Dispersed repetitive elements such as short interspersed nuclear elements (SINEs), long interspersed nuclear elements (LINEs) and human endogenous retroviruses (HERVs) account for

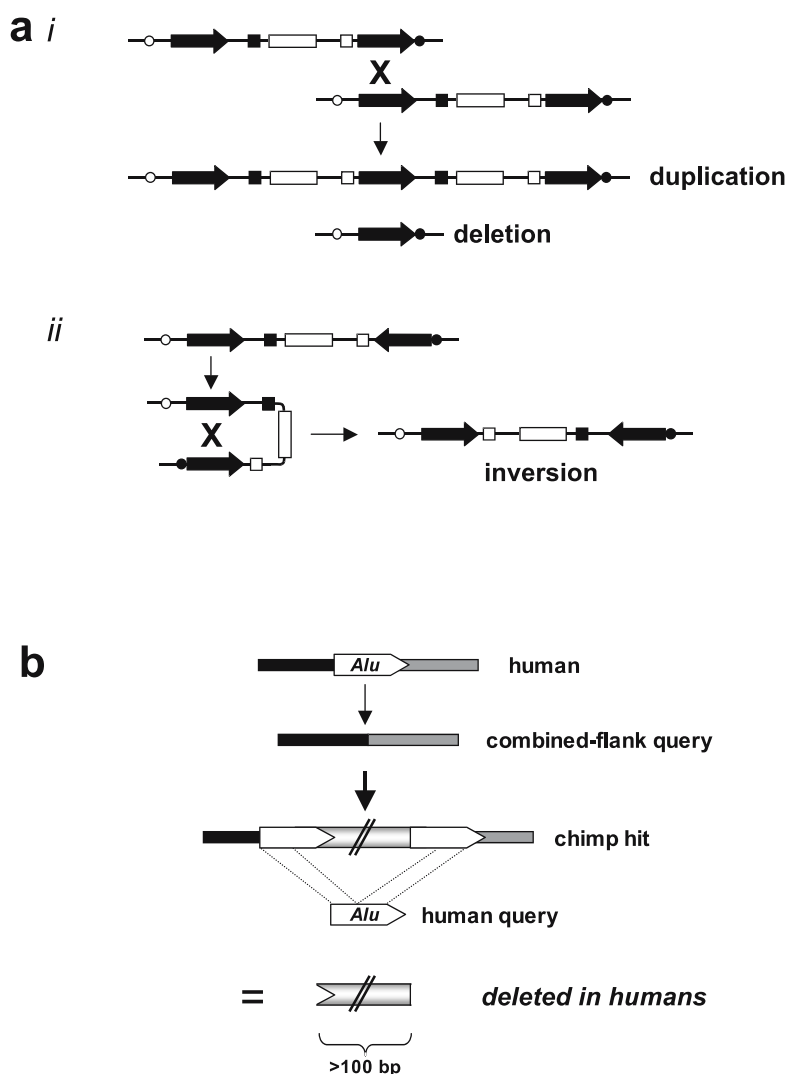


Fig. 6 Unequal crossover and the detection of *Alu*-mediated recombination. **a** Genomic rearrangements resulting from recombination between dispersed repeat sequences (*black arrows*). (i) Exchanges between non-allelic repeats in the same orientation can give duplications and deletions. Exchanges could occur within a molecule or between sister chromatids, as well as between homologous chromosomes, giving rise to intra- or inter-chromosomal rearrangements, respectively. (ii) Intra-molecular exchange between inverted repeats results in inversion. **b** *In silico* strategy used by Sen et al. (2006) to identify human-specific deletions arising from inter-*Alu* recombination. The 5' and 3' genomic sequences flanking all human *Alu* elements are extracted from the human genome reference sequence. A “combined-flank query” is constructed for each and the best match found in the reference chimpanzee genome. A longer chimp sequence containing all the information in the human *Alu* query signals a human-specific *Alu*-mediated deletion

around 40% of the genome (International Human Genome Sequencing Consortium 2001), whilst around 5% consists of segmental duplications of blocks > 1 kb in length with > 90% sequence similarity scattered in different genomic locations (Bailey et al. 2002). The advent of genome-wide studies such as HapMap has revealed the ubiquity of submicroscopic structural variation, and in particular copy number variation (CNV), even in the genomes of apparently healthy individuals (Freeman et al. 2006; Feuk et al. 2006; McCarroll et al. 2006). Indeed, many CNVs appear to exist as benign polymorphisms and a typical individual is thought to be hemizygous for 30–50 deletions of greater than 5 kb in size (Conrad et al. 2006). CNVs show significant clustering near segmental duplications, suggesting that NAHR may play a significant role in shaping normal patterns of variation in the human genome too (Perry et al. 2006).

5.1

***Alu* and L1 Elements as Mediators of Recombination**

With a copy number of more than a million and an average spacing of one per 3 kb, it is not surprising that the ~ 300-bp *Alu* element, the most abundant SINE in the human genome, has been directly associated with around 30 germline and at least 15 somatic rearrangements (Deininger and Batzer 1999). A recent genome-wide survey has attempted to assess the true impact of such events on genome dynamics (Sen et al. 2006). Nearly 500 *Alu/Alu*-mediated human-specific deletions have now been documented by comparing human and chimpanzee genomes (Fig. 6b). Around three-quarters of these deletions involve loss of less than 1 kb and over 70% appear to result from adjacent and directly orientated elements on homologous chromosomes. The deletions are positively correlated with regions of high *Alu* density and, since ~ 40% result from inter-*Alu* subfamily recombination, it seems that proximity is more important than sequence similarity in mediating ectopic exchange. That said, breakpoints do preferentially occur at the 5' end of the element in a region that shows strong conservation across all *Alu* subfamilies.

Similar germline events have been seen between human LINE-1 or L1 elements (Fitch et al. 1991; Burwinkel and Kilimann 1998) but in comparison to *Alu*-mediated events these are few and far between. This apparent difference has been the subject of some debate (Deininger and Batzer 1999; Sen et al. 2006). Element size and copy number are likely to contribute to this (full-length L1s are 6 kb but the majority are truncated to 800 bp, and there are ~ 520 000 L1s per genome), as might the difference in location (GC-rich regions for *Alu* versus GC-poor for L1). The paucity of L1-mediated germline events probably also reflects an ascertainment bias given that the lower density of L1s would result in larger rearrangements more likely to be deleterious.

Germline cases of *Alu*- and L1-mediated events include both deletions and duplications, consistent with their generation via unequal crossing over. However, chimeric elements accompanied by deletions have also been observed in cell-culture retrotransposition assays (Gilbert et al. 2005). These events could represent bona fide unequal crossing over between elements after insertion, or they might have arisen during insertion if the nascent cDNA joins to an upstream L1 by single-strand annealing and if resection and synthesis follow (see Gilbert et al. 2005). The latter mechanism might account for the greater dependence on proximity rather than sequence similarity between the two elements, and explains the clustering of breakpoints in deletions near preferred L1 endonuclease cleavage sites that initiate retrotransposition. A better understanding might be gained from characterisation of de novo events detected directly in sperm DNA although early attempts have been hampered by extremely low rearrangement rates (Hollies et al. 2001).

5.2

Lessons from Genomic Disorders

Most information on NAHR comes from the study of disease-related genomic rearrangements involving the proximal region of 17 p, and in particular those associated with the autosomal dominant disorders Charcot-Marie-Tooth disease type 1A (CMT1A) and hereditary neuropathy with liability to pressure palsies (HNPP) (for review see Lupski and Stankiewicz 2005). CMT1A is most often caused by overexpression of the *PMP22* myelin gene as a result of a heterozygous duplication mediated by a pair of 24-kb-long low copy repeats (LCRs) separated by some 1.4 Mb. Eighty to ninety percent of the milder and thus less frequently diagnosed HNPP cases result from the heterozygous deletion of the same interval (Chance et al. 1994). Studies of CMT1A/HNPP patients and of de novo duplications detected in sperm DNA have shown that exchange points within these LCRs tend to cluster into an interval of less than 700 bp, at the centre of which lies a 456-bp stretch of perfect identity shared by the two LCRs (Lopes et al. 1999; Reiter et al. 1998; Han et al. 2000). Less frequent events also map to stretches of identity greater than 200 bp in length, supporting the idea of a minimal efficient processing segment or MEP required for human ectopic recombination, similar to that noted for mouse cultured cells (132–232 bp—Liskay et al. 1987; Waldman and Liskay 1988). Patchy gene conversion often accompanies these ectopic exchanges, and it has been suggested that the DSB model may be equally applicable to unequal crossover as to allelic recombination (Lopes et al. 1999).

At the molecular level the CMT1A/HNPP deletions and duplications appear to be fully reciprocal. However, it is emerging that there may be sex-dependent differences in the frequency of deletions relative to duplications as well as differences in the predominant mechanism involved. For a limited number of individuals, the parental origin and nature of the rearrangement

have been determined from SNP and microsatellite data (Lopes et al. 1997, 1998, 1999). Over 90% of all CMT1A duplications are paternally derived and most appear to be generated by unequal meiotic exchange between homologous chromosomes. In contrast, the rarer maternal CMT1A duplications are as likely to be intra-chromosomal as inter-homologue events. Comparable data on HNPP deletions are scarce, but of just four de novo events only one was of paternal origin. As yet no attempt has been made to directly compare the frequencies of duplications and deletions in sperm DNA. Of the three maternal HNPP deletions, only two were informative for mechanism and both involved intra-chromosomal events that could either have been sister chromatid or intra-molecular rearrangements.

5.3

Relationship with Allelic Exchange

The clustering of unequal exchanges into small intervals within LCRs is not restricted to CMT1A/HNPP but appears to be a common feature of genomic disorders (Shaw and Lupski 2004). Since these clusters are remarkably similar in size to allelic crossover hotspots, it raises the question of whether ectopic hotspots are also hotspots for allelic exchange. This has now been directly addressed using sperm DNA approaches in the β -globin gene region (Holloway et al. 2006). This region is prone to ectopic exchange between a pair of homology blocks shared by the δ - and β -globin genes, giving rise to clinically recognised haemoglobin Lepore deletions that carry a δ - β fusion gene and anti-Lepore duplications carrying the reciprocal β - δ fusion gene (Efremov 1978). The region also contains the first ever provisionally identified allelic recombination hotspot in the human genome (Chakravarti et al. 1984).

The allelic hotspot was originally localised by LD analysis to an interval of over 9 kb (Chakravarti et al. 1984), but high-resolution characterization of de novo sperm crossovers revealed a typical, albeit very active, ~ 1.2 -kb-wide hotspot that just extends into the region of the β -globin gene that shares 570 bp of homology with the δ -globin gene (Holloway et al. 2006). Using a physical selection strategy to isolate and validate de novo deletions, the frequency of Hb Lepore deletions in sperm was found to be ~ 100 -fold lower than the rate of allelic exchange. In total, only four such deletions were identified and none involved inter-homologue exchange, although all were products of recombination between homologous δ and β sequences. Most importantly, the exchange points consistently avoided the crossover hotspot (Fig. 7). The same was true for a further six authentic but non-Lepore deletions presumed to have arisen via a non-homologous end-joining (NHEJ) pathway (Moore and Haber 1996). Thus, at the β -globin gene at least, there is no evidence that the allelic crossover hotspot drives any form of ectopic exchange (Holloway et al. 2006).

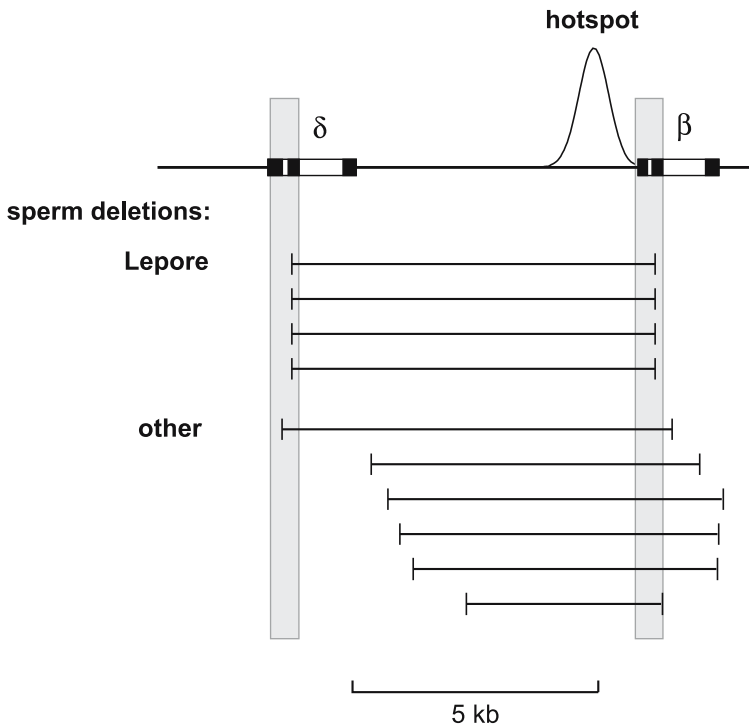


Fig. 7 Relationship between allelic and ectopic recombination at the β -globin allelic crossover hotspot. The hotspot is located upstream of the β -globin gene and just extends into a homology block shared with the δ -globin gene (grey shading). Unequal exchange between these homology blocks leads to Hb Lepore deletions. Physical enrichment of very large amounts of sperm DNA allowed the recovery and validation of the de novo deletion molecules shown, including four Lepore deletions and six other deletions not arising by homologous recombination. The Lepore breakpoints were all displaced away from the hotspot and occurred at an extremely low frequency (3×10^8 per sperm compared with a crossover frequency of 1.5×10^{-3} over the entire hotspot and of $\sim 3 \times 10^{-6}$ within the β -globin homology block). Likewise, other deletions were rare (frequency 4×10^{-8}) and had breakpoints distal to the hotspot. Data taken from Holloway et al. (2006)

5.4

Copy-Number Change Within Gene Families

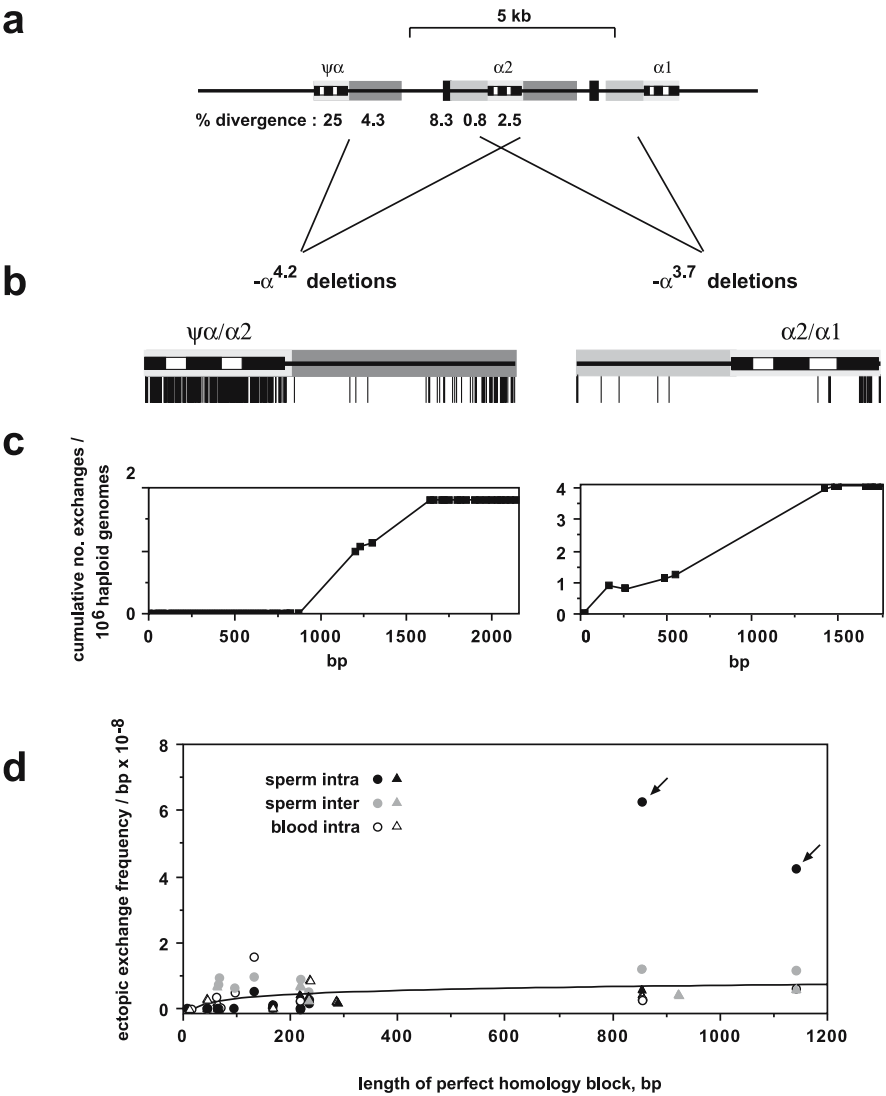
The extremely low frequency of deletion events between the δ - and β -globin genes has prevented further detailed analysis of ectopic exchange between these locally repeated DNA sequences. However, further insight into the dynamics of copy-number change within gene clusters can be gleaned from the duplicated α -globin genes. The $\alpha 1$ and $\alpha 2$ genes are embedded within two homologous segments of DNA that can be subdivided into several homology blocks with different degrees of sequence identity (Higgs et al. 1984). Ectopic

Fig. 8 Ectopic exchange in the α -globin gene region. **a** The α -globin genes and pseudo-gene plus associated homology blocks with like shading denoting similar sequences and with levels of sequence divergence between each homology block and its paralogue to the right shown *below*. **b** The two types of deletion analysed by single DNA molecule methods in sperm and blood. The locations of SNPs and paralogous sequence variants (PSVs) are marked by *lines below*. **c** Cumulative frequency of unequal exchanges in sperm across homology blocks. **d** Exchange frequencies estimated for each interval of sequence identity, with the best-fit logarithmic curve after excluding outliers (*arrowed*) with rates likely inflated by mutational mosaicism. Data are shown for two men analysed (*circles, triangles*), with sperm intra-chromosomal, sperm inter-chromosomal and blood exchanges plotted separately. Whilst ectopic exchange points avoid regions of high divergence they are otherwise fairly randomly distributed across homology blocks. Data from Lam and Jeffreys (2006)

exchanges between these blocks have led to a wide variety of deletion chromosomes that carry a single copy of the α -globin gene (Higgs et al. 1989; Embury et al. 1980; Bowden et al. 1987), many of which have attained high frequency in malarial regions via selection (Flint et al. 1986; Hill 1992).

Using a similar approach to that used to isolate Lepore deletions, large numbers of de novo α -globin gene deletions have been characterised from genomic DNA (Lam and Jeffreys 2006). The vast majority consist of simple exchanges that map to intervals of sequence identity between the two homologous regions. In contrast to CMT1A/HNPP, the α -globin exchanges are only rarely accompanied by patchy gene conversion, and at an incidence similar to that seen for allelic recombination (Jeffreys et al. 2000, 2001). In contrast to allelic crossover, the deletions are not restricted to germline (sperm) DNA but are also detected in blood. Somatic deletions arise by a strictly intra-chromosomal/intra-molecular pathway of homologous exchange that can lead to considerable degrees of mutational mosaicism that can influence overall mutation frequency. This process also predominates in the germline and is presumed to occur during the pre-meiotic germ cell divisions. However, around one quarter of sperm deletions arise through interactions between homologous chromosomes and presumably occur specifically at meiosis. This contrasts with CMT1A in which paternal rearrangements arise almost exclusively via homologous exchange between chromosomes. Surprisingly, ectopic exchanges between the α -globin genes show only weak dependence on uninterrupted homology length, challenging the notion of MEPs (Fig. 8).

Although in their infancy, these studies are already allowing direct comparison between germline mutation rates and population frequencies, and provide new insights into levels of selection acting on copy-number variants in human populations (Lam and Jeffreys 2006). However, many questions still remain including the issue of reciprocity of duplication, the relationship between these ectopic exchanges and allelic recombination, and the identification of factors that directly influence rates and patterns of unequal exchange.



6

Concluding Remarks

Our current knowledge of human recombination hotspots has been derived from diverse experimental approaches, each with their own strengths and weaknesses. In the last few years, the genomics revolution, and in particular the HapMap project, has played an important part providing for the very first time a high-resolution genome-wide picture of crossover distribution that would be impossible to achieve using current sperm screening

methods. This population approach has also given us the only real opportunity to look at events occurring within the female germline at this level of resolution. Ultimately, however, conclusions from HapMap data are inferential and depend crucially on the validity of the population genetic models and algorithms used to interpret genotype data. Further understanding will undoubtedly require advances in population modelling to improve inference methods and the use of complementary genomic approaches such as the Human Epigenome Project (<http://www.epigenome.org/>). However, sperm and testis studies will remain the only truly experimental approach to the detailed mechanistic analysis of human meiotic recombination capable of directly analysing factors that influence patterns and rates of human recombination. HapMap data will be invaluable in guiding these studies.

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Meiotic Nondisjunction—The Major Cause of Trisomy 21

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Abstract Chromosomal aneuploidy is a substantial risk for the human species. In this chapter, the knowledge about the origin and mechanisms of nondisjunction in human trisomy 21 that has accumulated during the last 15 years is summarized. The first molecular correlate of nondisjunction in humans is altered recombination, meiosis I errors being associated with reduced recombination and maternal meiosis II errors with increased recombination between the nondisjoined chromosomes. Thus, virtually all maternal meiotic errors of chromosome 21 seem to be initiated in meiosis I. Advanced maternal age remains the only well-documented risk factor for maternal meiotic nondisjunction, but there is still a surprising lack of understanding of the basic mechanisms behind the maternal age effect.

Abbreviations

MI, MII	meiosis I, meiosis II
DS	Down syndrome
AD	Alzheimer disease
CHD	congenital heart disease
β -APP	β -amyloid precursor protein
SMC1 β	meiosis-specific cohesion
MAD2	mitotic arrest deficient
BUB1	budding uninhibited by benomyl
apoE	apolipoprotein E
MTHFR	methylenetetrahydrofolate reductase
MTRR	methionine synthase reductase
PS-1, PS-2	presenilin-1, presenilin-2
ULO	unilateral oophorectomy
OC	oral contraceptive
PCR	polymerase chain reaction

1

Introduction

Chromosomal aneuploidy is the leading cause of fetal death in our species. Around 50% of spontaneous abortions until 15 weeks of gestational age are chromosomally aneuploid, with trisomies accounting for 50% of the abnormal abortions (Hassold et al. 1980). Among 1000 spontaneous abortions cytogenetically diagnosed, 26 had trisomy 21, 5 had trisomy 18 and 10 had

trisomy 13 (Hassold et al. 1980). Because of the high incidence in newborns, autosomal trisomies 21 (1–2 : 1000), 18 (1 : 5500–8000) and 13 (1 : 12 000) have large individual and socioeconomic consequences (Hassold and Jacobs 1984). Our knowledge about the mechanisms underlying chromosomal nondisjunction in man is, however, surprisingly poor. Advanced maternal age remains the only well-documented risk factor in nondisjunction (Penrose 1933), but the biological mechanisms of this phenomenon are not well understood. The incidence of trisomy among all clinically recognized pregnancies varies from 2% at age of 20 years to about 35% at the age of 40 years.

1.1

Down Syndrome Phenotypes

The clinical presentation of Down syndrome (DS) is complex and variable. Several features occur to some degree in each individual with trisomy 21, including characteristic facial dysmorphology, a small and hypocellular brain, and the histopathology of Alzheimer disease (AD), which is present by the fourth decade. Invariably, individuals with DS are cognitively impaired, though the severity is highly variable. Hypotonia occurs frequently in newborns, and most have atypical dermatoglyphic features, though the specific subset of these is again individually variable. Trisomy 21 is also a risk factor for a number of diseases. For example, it is among the leading causes of congenital heart disease (CHD), some form of which occurs in 40%–50% of individuals with DS (Ferencz et al. 1989). The incidence of childhood onset leukemia and Hirschsprung disease are both significantly elevated in patients with trisomy 21. Health care guidelines for individuals with DS include more than 80 clinical features that occur more frequently than in the population at large (Cohen 1999).

Three critical points for this discussion arise from these basic observations: (1) the incidence of most features seen in DS is variable; (2) the severity of a given feature is highly variable; (3) none of the features diagnosed in DS is unique to people with trisomy 21. For “DS features” that also occur in euploid individuals, we assume that there is some shared etiology regardless of ploidy, but this has to be proven for any specific case. A central challenge of genetic research in humans is to precisely define the phenotype. This is especially critical in DS, which is a product of genetic effects on different cells, structures, and functions throughout development, many of which may have cascading effects to produce clinically observed phenotypic end points in a given individual with trisomy 21 (Potier et al. 2006). A first step in this process is to separate those effects of trisomy that disturb development from those that alter function of cells that have reached an end point of differentiation. These are obviously not independent concepts; any “developmental” perturbation derives from alternation of some function in a developing cell. However, understanding when trisomy causes a divergence from normal pat-

terns of development in a cell that exists only for a defined period during embryogenesis requires a different experimental approach than measuring how trisomy affects a steady-state function (e.g., a signaling or metabolic pathway, neuronal response to stimulation, etc.) in a terminally differentiated cell.

Indeed, the altered functions of a mature cell may have little or nothing to do with up-regulation of trisomic genes in that cell, but rather could reflect a developmental error caused by trisomy that has downstream consequences that affect function. That is, a specific phenotype may be a consequence of but not a direct product of trisomic gene expression (developmental versus functional effects) (Roper and Reeves 2006). Genetic evidence has implicated three proteins, the β -amyloid precursor protein (β -APP) and the two homologous presenilins (PS-1 and PS-2), in the etiology of Alzheimer's disease (AD). The gene APP, encoding for amyloid precursor protein on chromosome 21 is an example of a single dosage-sensitive gene involved in Alzheimer's disease acting by itself to produce a phenotype.

1.2

Historical Background

Most of our knowledge about chromosomal nondisjunction in man comes from the studies in trisomy 21, the most frequent of the autosomal trisomies in liveborns. With an incidence of 1–2:1000 in human populations (Hook 1981), trisomy 21 is the most common single cause of mental retardation. The clinical entity, that we know as Down syndrome, was described more than a century ago (Down 1866), but it was not until 1959 that the etiology was discovered to be an extra chromosome 21 in all cell nuclei (Lejeune et al. 1959). The condition is usually the result of malsegregation (nondisjunction) of chromosome 21 in meiosis in either oogenesis or spermatogenesis.

The origin of nondisjunction in trisomy 21 was since the early 1970s studied using chromosomal short-arm heteromorphisms (Grouchy 1970; Juberg and Jones 1970), which by different staining techniques were informative in as many as 75% of the cases (Mikkelsen et al. 1980). The results of the major cytogenetic studies indicated that the origin of the extra chromosome was maternal in about 80% of the informative cases and paternal in about 20% (Juberg and Mowrey 1983; Hassold and Jacobs 1984). With the recombinant DNA technology a new set of tools became available to the study of origin and mechanisms of chromosomal abnormalities using DNA polymorphism analysis. In the beginning this kind of analysis used chromosome 21-specific DNA probes to detect restriction fragment length polymorphisms (Davies et al. 1984). The development of the polymerase chain reaction (PCR) amplification technique (Saiki et al. 1988) enabled the identification of novel and highly informative classes of DNA polymorphisms in the human genome, the so-called microsatellites or simple sequence repeat (SSR) polymorphisms

(Weber and May 1989; Litt and Luty 1989; Economou et al. 1990). Especially the studies of the multi-allelic and easily typable microsatellites have contributed to nondisjunction research in the more recent years (Petersen et al. 1991).

1.3

Segregation of Chromosomes in Mitosis and Meiosis

The alternative modes of cell division in mitosis and meiosis play different roles of particular interest in the life cycle of diploid organisms, such as animals and humans. While the chromosome number is kept strictly constant by mitosis in the diploid body cells, as well as in the mitotic germ line, it is reduced to a half by meiosis in the generation of male or female haploid germ cells. The fidelity of chromosome segregation relies on the mechanical properties of the spindle apparatus on the one hand and the connectivity of corresponding centromeres on the other. While the components of the spindle apparatus are identical in mitosis and meiosis, or nearly so, the characteristic differences in meiosis primarily concern the means of coordinating the centromeres to be segregated by division.

The spindle apparatus is mainly composed of microtubules and associated proteins, which together develop a high potential of self-organization. Parallel microtubules tend to associate in bundles, and these are termed spindle fibers. During nuclear division, the microtubules originate from two organizing centers at the spindle poles, as determined by the so-called centrosomal proteins. The free dynamic ends can encounter different targets in the cell by chance, which leads to some functional differentiation. If oppositely oriented microtubules meet between the poles, force is generated between these polar spindle fibers to push the poles further apart. If the dynamic ends of other microtubules encounter a centromere at a chromosome, these become attached to the kinetochore, forming a chromosomal spindle fiber¹. Mechanical force is generated towards the pole, and if the centromere is free to move, the microtubules are shortened accordingly by depolymerization at the kinetochore. However, these pulling forces cancel out by the simultaneous attachment of pairwise connected centromeres to both poles.

The peculiar metaphase plates in mitosis or meiosis are characterized by the temporary immobilization of all the chromosomes in equatorial rings around the spindles, due to their bipolar attachment at this stage. During mitosis, each chromosome consists of two sister chromatids, which stem from the preceding round of replication and are identical throughout their length.

¹ In fact, the chromosomes assume an active role in this encounter by nucleating special K-fibres at the kinetochores, which greatly facilitate the catching of other microtubules from the poles (see the introductory chapter by R. Egel, this BOOK). These K-fibres become especially important to self-organize the spindle during oogenesis, where many centrosomal functions are suspended (see Sect. 1.4).

Up to the metaphase stage, the sister kinetochores are facing in opposite directions from the firmly connected pericentromeric heterochromatin, which predisposes them to bipolar spindle attachment. In meiosis I, however, the sister kinetochores are fused as a functional unit, facing in the same direction from the chromatin. Instead, the partner centromere to be segregated to the other pole is provided by the indirectly connected homologous chromosome in a so-called bivalent.

Typically, bivalent formation requires the pairing of homologous chromosomes by synapsis and the exchange of DNA by crossing-over and chiasma formation (see various other chapters in this BOOK). In addition to the genetic effects of recombination, these chiasmata serve an important structural role in maintaining bivalent integrity until metaphase I of meiosis. This role crucially depends on sister chromatid cohesion, especially in the distal arms—at the far side of the connecting chiasmata, as seen from the homologous centromeres (Fig. 1A; see J.A. Susa and J.S. Rufas, this BOOK; K. Tanaka and Y. Watanabe, this BOOK). During meiosis I², sister chromatid cohesion is lost along the chromosome arms, but the sister centromeres remain connected until metaphase of meiosis II³. By then the sister kinetochores have been individualized and rearranged so as to face in opposite directions from the chromatin, which is similar to their organization in mitosis. Notably, the pairs of chromatids that are being segregated by sister centromeres in meiosis I and II are partly rearranged by recombination; thus, they are no longer sister chromatids throughout their entire length—only next to the sister centromeres and up to the nearest chiasma on either side.

The regular segregation of chromosomes in mitosis, as well as bivalents in meiosis I, critically depends on their bipolar spindle attachment. If any chromosome should be connected to one pole only, cells have a limited capacity to rectify this potentially hazardous situation, as mediated by the spindle attachment checkpoint system (see below). During meiosis, in particular, monopolar spindle attachment can accidentally arise from various causes. If two homologous chromosomes fail to synapse or do not acquire a single chiasma, either one tends to attach to a single pole as a univalent—more or less independently of the other (Fig. 1B). Incidentally therefore, both can be driven to the same pole, resulting in nondisjunction in meiosis I. Similarly, if sister chromatid cohesion at the distal arms fails prematurely, univalents can arise at meiosis I in spite of a preceding chiasma—especially if the only chiasma lies very close to a telomere (Fig. 1B). Moreover, one or two chiasmata very close to the centromeres can potentially lock the functional kinetochores of both homologues in a tightly connected cluster, all facing in the same direction from the chromatin (Fig. 1C), which could result in their being pulled to the same pole. Finally, sister chromatid cohesion could fail prematurely

² More precisely, at the metaphase–anaphase transition of meiosis I.

³ There is no DNA replication between meiosis I and meiosis II.

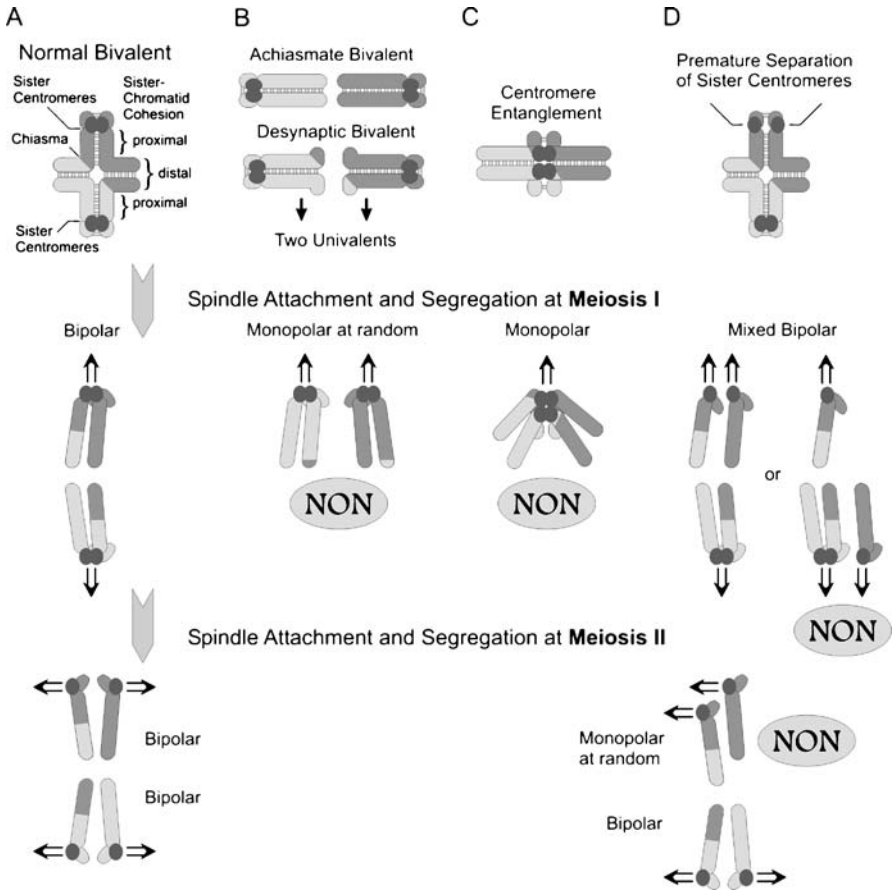


Fig. 1 The principal causes of nondisjunction in meiosis. For simplicity, each example displays a synapsed pair (bivalent) of homologous acrocentric chromosomes, with not more than one chiasma in the long arm. Metacentric bivalents, as well as more than one chiasma per bivalent, would result in more convoluted configurations. **A** A normal bivalent with a chiasma in the middle of the arm—and normal segregation in meiosis I and II—is shown for comparison. **B** Due to the lack of a chiasma or insufficient stability of a very distal chiasma, a bivalent can fall apart as two univalents. This results in a random assortment at meiosis I, with a 50% risk of nondisjunction (NON) at meiosis I, if both are moving to the same pole. **C** A chiasma very close to the centromere can lead to entanglement, interfering with proper segregation at meiosis I. **D** The premature separation of sister centromeres can lead to nondisjunction in meiosis I or II.—For the three examples of nondisjunction in meiosis I, the subsequent segregation in meiosis II is not shown

at the centromeres themselves (Fig. 1D), leading to independent assortment of sister centromeres at meiosis II—or even at meiosis I already, if cohesion is lost that early. The centromere entanglement caused by very close chiasmata may contribute to premature sister centromere separation. In addition

to such innate causes, exposure to environmental spindle poisons can also result in aberrant chromosome segregation, especially during mitosis.

1.4

Mammalian and Human Peculiarities in Meiosis

It is a characteristic feature of mammalian oogenesis that most of the first meiotic division in female is performed during fetal development, including synapsis and crossing-over, whereas chromosome segregation during MI and MII is delayed until ovulation and fertilization, a fact in contrast with the continuous process of spermatogenesis. This is especially interesting when later discussing risk factors for nondisjunction.

Animal spermatids and primary oocytes initially have typical centrosomes comprising pairs of centrioles and pericentriolar fibrous centrosomal proteins. The somatic cell-like centrosomes are partially or completely degenerated during gametogenesis. Centrosome reduction during oogenesis is due to complete degeneration of centrioles, which leads to dispersal of the pericentriolar centrosomal proteins, loss of replicated capacity of the spindle poles, and switching to acentrosomal mode of spindle organization. Oocyte centrosome reduction plays an important role in preventing parthenogenetic embryogenesis and balancing centrosome number in the embryonic cells. The male and female gametes degenerate centrosomes in a reciprocal manner so that after fusion their centrosomal components complement each other and generate a functional zygotic centrosome (Manandhar et al. 2005).

Both meiotic divisions of the mouse oocyte are asymmetric. Such asymmetry is ensured by the positioning of the spindle in the periphery of the large oocyte. The MI spindle generally forms in the center of the oocyte and migrates towards its periphery (Longo and Chen 1985; Maro and Verlhac 2002). The MII spindle forms in the periphery of the oocyte and is maintained close to the plasma membrane during the metaphase arrest. Our knowledge on the mechanisms of asymmetric divisions stems from investigations on mitotic cells (Betschinger and Knoblich 2004) where spindle positioning depends on interactions between the cell cortex and “astral” microtubules that connect the spindle poles to the cell cortex (Cowan and Hyman 2004). Oocytes lack centrosomes and the spindles, in turn, lack astral microtubules: alternative mechanisms must be at play to position the spindle within the oocyte. In mouse oocytes, spindle migration and anchoring require actin microfilaments but not microtubules (Longo and Chen 1985; Maro et al. 1986; Van Blerkom and Bell 1986; Verlhac et al. 2000; Leader et al. 2002; Maro and Verlhac 2002). Direct interactions between chromosomes and actin govern spindle positioning. In addition, chromosomes mediate cortical actin reorganization by an “at distance” effect. Brunet and Maro (2005) propose that in mouse and more generally in mammalian oocytes, chromosomes act as a “territory landmark” to organize both microtubules and actin microfila-

ments. This spatial control is essential to achieve the two asymmetric meiotic divisions that lead to the formation of a functional gamete (Brunet and Maro 2005).

Mitotic chromosome segregation is facilitated by the cohesin complex, which maintains physical connections between sister chromatids until anaphase. Meiotic cell division is considerably more complex, as cohesion must be released sequentially to facilitate orderly segregation of chromosomes at both meiosis I and II. This necessitates meiosis-specific cohesin components; recent studies in rodents suggest that these influence chromosome behaviour during both cell division and meiotic prophase. To elucidate the role of the meiosis-specific cohesin *SMC1 β* in oogenesis, Hodges et al. (2005) carried out meiotic studies of female *SMC1 β* -deficient mice. Their results provided the first direct evidence that *SMC1 β* acts as a chiasma binder in mammals, stabilizing sites of exchange until anaphase. Their observations support the hypothesis that deficient cohesion is an underlying cause of human-related aneuploidy (Hodges et al. 2005).

Dosage imbalance of whole chromosomes typically results in inviability. So, it is not surprising that, in most organisms, meiotic nondisjunction is a rare occurrence. In mammals, the frequency of meiotic errors seems to be higher than in other organisms; nevertheless in the most studied organism, the mouse, the overall incidence of aneuploidy among fertilized eggs does not exceed 1–2%. Human species provides a notable exception to this general rule. The overall rate of nondisjunction appears to be much higher in human eggs than in other animals. An estimated 10–30% of fertilized human eggs have the “wrong” number of chromosomes, with most of these being either trisomic or monosomic. The basis for the difference in incidence between our own and other species remains obscure (Hassold and Hunt 2001).

In humans, the ovulation cycle is connected to a decline in functional oocytes, not only due to those being ovulated, but also by additional ones becoming atretic. As women age, the decline in the size of the total oocyte pool is accompanied by a decline in the number of antral follicles that mature during each menstrual cycle. Mechanisms related to nondisjunction could, in theory, operate at any time between the formation of the oocyte pool—when the woman herself is in utero and meiosis I begins—and the completion of meiosis I at ovulation (Kline et al. 2000). Additionally, there appears to be a link between the incidence of trisomy and the speed of oocyte depletion. In their study, Kline et al. (2000) posited accelerated oocyte atresia⁴ as the underlying common pathway to trisomy and menopause, but they were not able to distinguish between this pathway and the equally plausible hypothesis that fewer oocytes were formed during fetal development in women who later conceived trisomic pregnancies (Kline et al. 2000).

⁴ The degenerated process by which oocytes and follicles perish without having been expelled by ovulation.

2

Spindle Assembly Checkpoint in Mammals and in Humans

Exit from mitosis in animal cells is substantially delayed when spindle assembly is inhibited, spindle bipolarity is disrupted, or when a monopolar spindle is formed. These observations have led to the proposal that animal cells have a spindle assembly checkpoint for the metaphase-anaphase transition that monitors bipolar spindle organization. However, the existence of such a checkpoint is uncertain because perturbations in spindle organization can produce unattached kinetochores, which by themselves are known to delay anaphase onset. In their study, Sluder et al. (1997) conclude that animal cells do not have a checkpoint for the metaphase-anaphase transition that monitors defects in spindle architecture independent of the checkpoint that monitors kinetochore attachment to the spindle. For dividing cells in which spindle microtubule assembly is not experimentally compromised, they proposed that the completion of kinetochore attachment is the event which limits the time of the metaphase-anaphase transition (Sluder et al. 1997).

In humans, if defects are detected, a signal is transduced to halt further progression of the cell cycle until correct bipolar attachment to the spindle is achieved. Two genes encoding conserved kinetochore-associated proteins (MAD2 and BUB1)⁵ are believed to be components of the checkpoint regulatory pathway. A failure in this surveillance system could lead to genomic instability that may underlie the increased incidence of aneuploidy in the gametes of older women. To explore this possibility, Steuerwald et al. (2001) determined the concentrations of these transcripts in human oocytes at various stages of maturation by RT-PCR technique. The results obtained following quantitative analysis suggested that these messages degrade as oocytes age. Potentially, this may impair checkpoint function in older oocytes and may be a contributing factor in age-related aneuploidy (Steuerwald et al. 2001).

3

Stages of Origin of Nondisjunction

3.1

Meiotic Stage—Indirect and Direct Studies

Meiotic recombination is generally suppressed across the centromere of eukaryotic chromosomes (Choo 1998). In the last years, a paradox has emerged regarding the relationship of centromere structure and its function. Most centromeric DNAs analyzed to date are composed of a remarkably complex array

⁵ These genes were first detected in budding yeast as mitotic arrest deficient (MAD2) and budding uninhibited by benomyl (BUB1).

of repeat structures. In contrast, analyses of neocentromeric DNA reveal that repetitive DNA is not a prerequisite for centromere activity. The ubiquity of repetitive sequences among diverse species at sites of primary constriction argues that there is a strong evolutionary link between centromere structure and function. Emerging repetitive structures at centromeric sites may be an important byproduct of a functional centromere which ensures that this site is an evolutionarily favored position in subsequent meiotic and mitotic lineages (Eichler 1999).

The centromeric heterochromatin on chromosome 21 has been evaluated by the fluorescence in situ hybridization (FISH) technique. It was found that the alphoid DNA sequence of pericentromeric regions of chromosome 21 was highly heteromorphic when a centromere specific probe was hybridized to these sequences (Verma et al. 1997).

Studies of meiotic stage of nondisjunction (meiosis I or II) in trisomy 21 by DNA polymorphism analysis were hampered by the lack of centromeric markers. Alphoid DNA polymorphisms specific for the human chromosome 21 centromere had been described (Jabs et al. 1991), but the informativeness of these markers was low, and they were not useful for routine nondisjunction studies. An alphoid DNA polymorphism was localized on top of the genetic linkage map of human chromosome 21 (Petersen et al. 1991; Jabs et al. 1991), giving an estimate (6 cM) of the genetic distance between the centromere and the closest pericentromeric DNA markers on the long arm of chromosome 21. The meiotic division error has been inferred on the basis of nonreduction/reduction to homozygosity at pericentromeric DNA polymorphic markers (Chakravarti and Slaugenhaupt 1987). Among the maternal errors, approximately 75% was attributed to errors in meiosis I and 25% to errors in meiosis II (Antonarakis et al. 1992; Mikkelsen et al. 1995; Yoon et al. 1996). Both maternal meiosis I and II errors are associated with increased maternal age (Antonarakis et al. 1992; Mikkelsen et al. 1995; Yoon et al. 1996). In a study of 200 families, each with a proband with cytogenetically diagnosed free trisomy 21, DNA markers were used to determine the meiotic stage of nondisjunction (Antonarakis et al. 1992). For this purpose, only markers that are at the centromere can be used, although pericentromeric markers can be used as a surrogate. The reduction to homozygosity of a given pericentromeric marker was interpreted to be the result of an error in meiosis II. When the meiotic stage of origin was determined with more than one pericentromeric marker, there was never a discrepancy between the results of different markers (Antonarakis et al. 1992). For example, if a mother of a maternally inherited trisomy 21 has alleles AB for a given pericentromeric marker, and the proband AAC, then the error was assigned to meiosis II. Alternatively, if the offspring of this particular example has alleles ABC for this pericentromeric marker, then the error was determined to have occurred in meiosis I. It is important to note that a mitotic error very early in either the zygote or the few-cell embryo will result in a scoring of DNA markers indistinguishable from a meiosis

II error; however, if a DNA marker along the long arm of the chromosome does not show reduction to homozygosity, then the possibility of a mitotic error is negligible, since mitotic cross-overs are rare. Undoubtedly, errors in the determination of the meiotic stage of nondisjunction have been made with both the DNA polymorphisms and the cytogenetic heteromorphisms, since neither group of polymorphisms marks the centromere of chromosome 21. In the case of DNA polymorphisms, we can roughly estimate that the error rate in the prediction is approximately 6%–10% (if it is assumed that the rate of crossing-over is the same in normal meiosis and in meiosis that leads to nondisjunction), since this is the genetic distance of the pericentromeric markers from the centromere in the CEPH (Centre d'Etude du Polymorphisme Humain) families (Jabs et al. 1991). However, it has been shown that there is reduced recombination in chromosomes 21 that undergo nondisjunction; therefore the error rate in the determination of meiotic stage of nondisjunction by using DNA polymorphisms could be smaller than the predicted 6%–10%. It appears that the error rate is proportional to the genetic distance of the most proximal marker to the centromere. This distance is not known in the absence of centromeric markers or short-arm markers. Therefore, the "reduced recombination" rate mentioned here is deduced from activity along the explored long arm of chromosome 21 and it is extrapolated that the rate is also reduced within the unexplored centromeric interval. Laurent et al. (2003) have developed proximal and short-arm markers and demonstrated that recombination is indeed repressed in normally disjoining chromosome 21 and also in nondisjoining chromosome 21. This finding has an impact on reducing the error rate in determining meiotic stage of nondisjunction but also in showing that absence of recombination, rather than potential centromeric recombination events leading to premature sister separation, is quantitatively the main cause of nondisjunction. In the case of chromosomal heteromorphisms the error rate cannot be estimated, since the extent of recombination on the short arms of acrocentric chromosomes is unknown. In a subset of the families that were studied, the data obtained by cytogenetic heteromorphisms and the data determined by DNA polymorphisms were compared (Antonarakis et al. 1992). The karyotypes of 31 families were scored for short-arm heteromorphisms after staining of the chromosomal slides either with quinacrine or, for nucleolar organizing regions, with silver (Mikkelsen et al. 1980). Only results that were considered to unequivocally indicate a specific meiotic origin were compared with the data from DNA polymorphisms. There were several discrepancies between the two methods. First, there were erroneous parental assignments by chromosomal heteromorphisms in three families. In the remaining 26 families, in which the two methods agreed on the parental origin, there were three discrepancies with regard to the determination of the meiotic stage of nondisjunction (Antonarakis et al. 1991).

In their study, Laurent et al. (2003) developed polymorphic microsatellite markers embedded within the duplicated most proximal sequences of the

long and the short arm of chromosome 21 both in male and female but not in the most proximal 21q region in female, therefore the repression of recombination observed near centromeres in female was not estimated. Extreme size variations of the alpha-satellite I blocks transmitted in these families and deduced from quantitative FISH analysis were not correlated with the inter-individual variations of recombination activity observed in the pericentromeric region. None of 28 families with a trisomy 21 child previously associated with a nullitranstitional meiosis I nondisjunction event presented a recombination exchange across the centromere. This confirms that for this group of errors, the lack of recombination is the primary susceptibility factor, not abnormal recombination in the centromeric region (Laurent et al. 2003).

In a population-screening study Lo et al. (1999) demonstrated that extreme reduction of chromosome-specific alpha-satellite is unusually common in chromosome 21, with a prevalence of 3.70% compared to $\leq 0.12\%$ for each of chromosome 13 and 17, and 0% for other chromosomes. No analphoid centromere was identified in >17 000 morphologically normal chromosomes studied.

Information on recombination has also been obtained using indirect, genetic methods. Tease et al. (2002) used an immunocytological approach, based on detection of foci of a DNA mismatch-repair protein, MLH1, on synaptonemal complexes at prophase I of meiosis, to provide the first direct estimate of the frequency of meiotic recombination in human oocytes. At the stage of pachytene, the stage of maximum homologous chromosome pairing, a mean of 70.3 foci (i.e., cross-overs) per oocyte was found. The numbers and positions of foci were determined for chromosomes 21, 18, 13, and X. A mean of 1.23 foci (61.5 cM) was yielded for chromosome 21. The foci were almost invariably located interstitially and were occasionally located close to chromosome ends (Tease et al. 2002).

In contrast to the situation in human female germ cells, meiotic recombination in human male germ cells has been investigated through both indirect and direct means. Classically, the direct approach has made use of chiasmata (i.e., the cytologically visible consequences of crossing-over) to determine the numbers and distributions of cross-overs at diakinesis/metaphase I (Hultén 1974; Laurie and Hultén 1985). The number and distribution of foci in pachytene spermatocytes mirror those of chiasmata at metaphase I in mouse spermatocytes (Anderson et al. 1999). The crucial role that the MHL1 protein plays in crossing-over is additionally supported by the observation that chiasma formation is essentially abolished in mice with a knockout of the *MLh* gene (Edelman et al. 1996; Woods et al. 1999). In their study, Tease et al. (2002) indicated that the level of crossing-over in female germ cells is 1.4 greater than that in male germ cells⁶. This is in comparison to the hu-

⁶ The regional differences of crossover rates in male and female human meiosis are more fully discussed by C. May, T. Slingsby and A.J. Jeffreys (this BOOK).

man female germ cell:human male germ cell estimate of 1.6:1 from a study of genetic map lengths (Broman et al. 1998). It is not yet clear whether cross-over interference, a crucial factor in the determination of the numbers and distributions of cross-overs, varies between human male meiosis and human female meiosis (Broman and Weber 2000; Lynn et al. 2000). One possible explanation for the sex-related difference in recombination frequency is that the strength of interference is greater in spermatocytes, hence giving the reduced number of cross-overs in spermatocytes, in comparison to that in oocytes. An alternative possibility can be envisaged from the observation that pachytene chromosomes in oocytes are approximately double the length of those in spermatocytes (Bojko 1983; Wallace and Hultén 1985) that is, the female genome has much longer physical platform for meiotic recombination than the male genome (Tease et al. 2002).

3.1.1

Chiasma Configuration

Under normal meiotic conditions, the presence of a single chiasma, regardless of its location, may be sufficient for proper chromosome segregation. However, as the oocyte ages, a decay or breakdown in the meiotic apparatus (e.g., a spindle component or sister chromatid cohesion protein) may occur, disturbing the meiotic process. At this point, certain exchange configurations may be more likely to undergo improper segregation and nondisjunction. In this manner, as the age of a woman increases, so too does her chance of a meiotic disturbance. It is unclear if these differences in overall patterns of recombination are due to the prevalence of one specific type of chromosomal exchange on chromosome 21 or rather a shift in the global exchange patterns for the chromosome. Genetic maps are a composite of the achiasmate, single and multiple tetrads and are not easily separated into each component part. Although genetic maps can represent locations and clusters of exchanges, they cannot, for example, determine if the proximal clustering found among the MII trisomy 21 cases is due primarily to single or higher-order exchanges occurring in this region. To answer this type of question, other methods must be employed.

In most organisms, a chiasma can occur anywhere along the euchromatin of the chromosome arm. The probability of a chiasma, however, is not a constant value across the chromosome, but rather follows specific patterns of placement. In human males, exchange patterns have been obtained from direct chiasma counts of spermatocytes. Similar frequency and distribution studies have been difficult to obtain for females, due to technical problems in procuring appropriately staged oocytes for study.

Recently, analytical methods to estimate the array of meiotic tetrads that give rise to a sample of meiotic events based upon the cross-over frequencies observed in family data have been presented (Lamb et al. 1997). The chromo-

some of interest is divided into several intervals, and the recombinant status (the observed number of recombinations and the interval where each is located) for each chromosome is determined. Chromosome exchange distributions are then estimated from these recombination patterns using maximum likelihood methods. Such an analysis can be applied to both male and female samples, circumventing many technical problems inherent to cytological chiasma counting. In addition, the frequency and distribution of exchanges from abnormal meiotic events, like nondisjunction, can be analyzed by these methods. These techniques were employed to examine populations of chromosome 21 where either MI or MII nondisjunction has occurred, extending the work of previous recombination-based studies (Lamb et al. 1997). A more extensive set of chromosomal markers were utilized to provide more complete coverage along chromosome 21, particularly near the telomere (Lamb et al. 1997). The estimated exchange patterns obtained from the nondisjoined populations were then compared with the exchange patterns of a sample of normal chromosome 21 female meiotic events to better understand the significant differences in genetic maps observed between the nondisjunction populations and the normal population (Lamb et al. 1997). Of specific interest was the bimodal distribution of exchange reflected by the telomeric cluster in the MI nondisjunction population and the proximal cluster in the MII nondisjunction population. The results indicated that susceptible meiotic tetrads were associated with the distance between the centromere and the closest meiotic exchange.

Pericentromeric exchanges appear to predispose a tetrad to an MII nondisjunction. On the other hand, if the closest exchange is near the telomere, a great distance from the centromere, an MI nondisjunction susceptibility appears to be established. The frequencies of each type of recombinant event were calculated for maternal MI and MII trisomic events, as well as for normal female meiotic events. An "observed recombinant" resulting from an exchange occurring during the four-strand stage of meiosis is based on more information when two chromatids are contributed by a parent due to nondisjunction than when only one chromatid is contributed after normal meiosis. When two chromatids are contributed, a transition from heterozygosity to homozygosity (or vice versa) between genetic markers indicates an observed recombinant event. When only one chromatid is contributed, a switch in parental phase between genetic markers indicates an observed recombinant. The overall frequency and placement of exchanges along the tetrad were estimated for each population.

The mitotic cases constitute a separate group of chromosomal errors. Irrespective of this, by definition, there are no achiasmate tetrads in the MII population, as such cases are classified as mitotic in origin. Examination of the overall exchange distribution supports the observations drawn from previous studies of the genetic maps: the distribution of recombination for chromosome 21 involved in either maternal MI or MII nondisjunction is dif-

ferent from normal female meiosis. A greater proportion of exchange was observed along the distal region of the chromosome in the MI nondisjunction population, while increased proximal exchange was found for the MII nondisjunction sample (Lamb et al. 1997). Meiotic events with at least one exchange were examined to determine if certain configurations were susceptible to nondisjunction. The placement of the single exchange event was found to account for nearly all of the deviation in the trisomic distributions. The MI population of single exchanges showed a strong shift towards the telomere. More than 80% of all single exchanges were located in the telomeric third of the chromosome, compared with only 40% of those that segregated normally. In contrast, the distribution of single exchanges for the MII population was shifted in the opposite direction, towards the centromere. Nearly 63% of single exchanges occurred in the proximal third of the chromosome for this population, compared to only 35% in the normal population. To investigate this centromeric shift more closely, the most proximal interval on the chromosome was subdivided into four smaller segments. The location of single exchange events was first examined. In the normal population, nearly all of the exchanges were located at the distal end of the interval. A marked shift towards the centromere was observed when the MII population was examined; exchanges were widely distributed across the interval and, in general, located more closely to the centromere than in the normal population. Pericentromeric exchange therefore, appears to be a distinguishing feature in the MII nondisjunction. Unlike single exchanges, the distribution of double exchanges in both trisomic populations roughly approximated the distribution of the normal individuals.

An additional method to compare the exchange distributions identifies the location of the most proximal exchange for each meiotic event among the different populations. This serves as a measure of the distance between the centromere and closest meiotic exchange. Additionally, this type of comparison combines the contributions from both single and double exchange events. For this analysis, the chromosome was divided into thirds (proximal, medial, and distal), and the location of the most proximal exchange was identified. From these values, odds ratios to measure the association between exchange placement and nondisjunction were constructed. Medial region was used as the "referent" (non-susceptible) region. If the most proximal exchange in a meiotic tetrad occurred in the proximal region of the chromosome, that tetrad was ~ 2.8 times as likely to undergo an MII nondisjunction than if it were in the medial region. In contrast, if the most proximal exchange was in the distal region of the chromosome, that tetrad was ~ 4.9 times as likely to undergo an MI nondisjunction than if the exchange occurred in the medial region. Theories involving chiasma placement and disjunction are consistent with these findings. Hawley et al. (1994) proposed that the reduced exchange rates observed for chromosomes nondisjoined at MI reflected the tendency for certain configurations of exchange, specifically single distal exchanges, to un-

dergo nondisjunction more frequently than others (Hawley et al. 1994). These distal exchanges less effectively link homologues and orient each kinetochore to opposing spindle poles. Even so, in a fully functional oocyte, these susceptible configurations would still disjoin normally. Hawley proposes, however, that the ability of a woman's oocyte to form normal spindles diminishes as the age of the woman increases. Under these unpaired conditions, the weakness of susceptible exchanges would become evident. In the presence of a damaged spindle, distally linked homologues, would be less likely to orient correctly at the metaphase plate than their counterparts with proximal exchanges. Subsequently, these bivalents, along with achiasmate tetrads, would be ejected from the spindle and, as tetrad, randomly migrate into the secondary oocyte or polar body (Lamb et al. 1997).

Models have also been advanced to explain the increased proximal exchange observed in chromosomes that undergo MII nondisjunction. Possibly, proximal chiasmata predispose to "chromosome entanglement" at MII, with the bivalent being unable to separate, passing intact to the MII metaphase plate (Lamb et al. 1996). Upon MII division, the bivalent divides reductionally, resulting in a disomic gamete with identical centromeres. In this manner, proximal recombination, an event which occurred during MI, is resolved and visualized as an MII error. This model also depends upon some type of age-dependent meiotic disturbance in addition to a susceptible meiotic tetrad for the occurrence of a nondisjunction event. An alternate theory proposes that the resolution of proximal chiasmata leads to premature sister chromatid separation just prior to anaphase I (Koehler et al. 1996). Resolution of chiasmata requires the release of sister chromatid cohesion distal to the site of the exchange. Attempts to resolve chiasmata that are very near the centromere could result in premature separation of the chromatids. If the sister chromatids migrate to a common pole during MI, they have a 50% chance of randomly travelling into the same product of meiosis during MII, resulting in an apparent MII nondisjunction. Studies of nondisjunction in humans have provided preliminary support for this model. Sherman et al. (2005) investigate the proneness of pericentromeric cross-overs to nondisjunction at MI. They discuss "entanglement" and premature sister centromere separation as possible events with the first to appear as more persuasive. In model systems, univalents can occasionally attach to both poles simultaneously. This can lead to considerable stretching of the centromeres at MI, but very rarely are sister centromeres actually split apart in such a case. A chiasma directly adjacent to the compact domains of pericentromeric heterochromatin can constrain the orientation of non-sister kinetochores in an unfavorable manner, increasing their risk to be engaged in a persistent monopolar spindle attachment, which can lead to nondisjunction.

A common thread that runs through many of these models requires summation of specific meiotic events to occur before a nondisjunction takes place. Although specific patterns of exchange may be susceptible to nondisjunc-

tion, on their own these patterns are not sufficient to ensure mal-segregation. Some additional meiotic agitation (abnormal spindle formation, premature release of sister chromatid adhesion, hormonal imbalance, etc.) is required for nondisjunction. As there is no maternal age effect on meiotic exchange, it is this meiotic agitation that must be dependent on maternal age. Whatever mechanism is responsible for the nondisjunction event, it appears that specific patterns of chromosomal exchange are associated with chromosome 21 aneuploidy. The trisomic population, however, does contain a subgroup of tetrads which mirror the normal population in number and placement of exchanges. For this group, circumstances in the oocyte, unrelated to genetic recombination, may be responsible for chromosome mal-segregation. The compromised micro-circulation hypothesis of Gaulden suggests that nondisjunction arises from a series of cascading events (Gaulden 1992). Initially, a poorly developed microvasculature is created around the ovarian follicle, resulting in a reduced blood flow through the area. Gaulden suggests this could be due to a hormonal imbalance occurring during follicular development.

3.2

Mitotic Stage

About 5% of cases of trisomy 21 are probably due to mitotic (postzygotic) nondisjunction of a chromosome 21 in the early embryo, as determined by pericentromeric DNA markers and the lack of observed recombination along the entire long arm of chromosome 21 (Antonarakis et al. 1993; Mikkelsen et al. 1995; Yoon et al. 1996). The mitotic errors are not associated with advanced maternal age and show no preference in the parental origin of the duplicated chromosome 21 (Antonarakis et al. 1993). Mosaicism with a normal cell line occurs in about 2%–4% of Down syndrome newborns (Hook 1981). DNA polymorphism analysis with mosaic trisomy 21 probands showed that the majority of cases resulted from a trisomic zygote with mitotic loss of one chromosome (Pangalos et al. 1994). Mitotic errors occur during the cleavage stage with the majority identified during the first and second cell divisions prior to the activation of the embryonic genome. More than 80% of the mosaic aneuploid embryos showed mitotic gains and losses with different proportions of diploid, monosomic and trisomic cells, suggesting that the original zygote was diploid and that both gametes contained only one copy of each chromosome 21. Mosaic aneuploid embryos could combine meiotic and mitotic cell division errors, suggesting that the observed diploid cells were a result of anaphase lag of chromosome 21 in an aneuploid zygote and that one of the gametes had two copies of chromosome 21 resulting in the aneuploid conception. However, the majority of these mosaic embryos were originally diploid conceptions (Katz-Jaffe et al. 2004).

The difference in the nature of chromosome 21 errors between preimplantation embryos and first and second trimester fetuses suggest that mi-

totic nondisjunction of chromosome 21 is non-viable. Even though there are clinical reports of chromosome 21 mosaic pregnancies and confined placental mosaicism (Kalousek and Vekemans 1996; Nicolaidis and Petersen 1998) these are considerably less in number than the frequency of chromosome 21 mosaicism from mitotic cell division error observed in the early embryo. One possible explanation for the non-viability of embryos with chromosome 21 mitotic error to successfully implant and maintain a pregnancy could be due to imprinting errors. Imprinted genes function from just one allele, either maternal or paternal, while methylation silences the other allele during early embryo development (Vastag 2001; Butler 2002). It is conceivable that mitotic cell division errors of chromosome 21 cause aberrant expression of imprinted genes in the embryo that is lethal at or post-implantation. Given that all the other chromosomes, excluding Y, contain a larger number of imprinted genes (Butler 2002), it is likely that mitotic cell division errors in any chromosome are also associated with non-viability.

4

Parental Origin and Parental Ages

Two large collaborative studies used multiple DNA polymorphisms spanning the long arm of human chromosome 21 to determine the parental origin of nondisjunction in trisomy 21 (Antonarakis et al. 1991; Sherman et al. 1991). These studies estimated that only 5% of the cases (of a total 304 families studied) had paternal origin and attributed the difference from the cytogenetic studies to an increased accuracy of the DNA polymorphism analysis, as demonstrated by erroneous cytogenetic determinations in a subgroup of

Table 1 Origin of nondisjunction in human trisomy 21 by DNA polymorphism analysis

Origin*	Number of cases	%	Meiotic recombination
Maternal	732	90.7%	
MI	556	68.9%	Reduced
MII	176	21.8%	Increased
Paternal	44	5.5%	
MI	17	2.1%	Reduced
MII	27	3.3%	
Mitotic	31	3.8%	
"Maternal"	17	2.1%	
"Paternal"	14	1.7%	

* MI = meiosis I, MII = meiosis II, "Maternal" and "Paternal" refer to parental origin of the chromosome that was duplicated by postzygotic nondisjunction. Data from Antonarakis et al. (1993), Lamb et al. (1996), Savage et al. (1998)

Table 2 Mean parental ages by origin of nondisjunction in population-based newborn studies

Origin*	<i>n</i>	Maternal age	Paternal age
Maternal			
MI	145	30.1	32.0
MII	50	31.2	33.3
Paternal			
MI + MII	16	25.6	29.9
Mitotic	12	28.2	30.5

* MI = meiosis I, MII = meiosis II. Data from Mikkelsen et al. (1995) and Yoon et al. (1996)

families. More recent population-based studies show 5–9% paternal meiotic errors (Mikkelsen et al. 1995; Yoon et al. 1996). The latest results of the major groups studying nondisjunction in trisomy 21 are summarized in Table 1. A significant difference in mean maternal ages was found between cases of maternal origin and those of paternal origin in a molecular study (Petersen et al. 1993). This indicates that the maternal age effect in Down syndrome is confined to maternal nondisjunction, and does not provide evidence for a relaxed selection against trisomic fetuses in older women, as suggested on the basis of the cytogenetic studies where there was no evidence for a difference in mean maternal age between trisomy 21 cases of maternal and paternal origin (Aymé and Lippman-Hand 1982; Stein et al. 1986). A factor associated with aging of the oocyte therefore seems responsible for the maternal age effect in Down syndrome. The mean parental ages stratified by origin of nondisjunction in population-based newborn studies are shown in Table 2.

5 Parental Nondisjunction

In paternal nondisjunction of chromosome 21 there is an excess of meiosis II errors in contrast to maternal nondisjunction, where meiosis I errors predominate⁷, as indicated by DNA polymorphisms. The mechanisms associated with paternal nondisjunction are therefore likely to differ from those associated with maternal nondisjunction. The paternal age effect had been a matter of big controversy before the DNA studies and the issue was raised again by a study showing an effect of donor (paternal) age on the incidence of trisomy 21 after artificial insemination with frozen donor spermatozoa,

⁷ It is well known that there is a male-specific increase of crossovers close to the telomere(s) (Hultén 1974; Laurie and Hultén 1985; see C. May, T. Slingsby and A.J. Jeffreys, this BOOK).

independent of maternal age, but the parental origin of trisomy 21 was not determined (Thepot et al. 1996). There is a well-known increased male:female sex ratio (around 1.15) in liveborns with Down syndrome (Huether et al. 1996). This effect is restricted to free trisomy 21 and does not extend to translocations, suggesting that the increased ratio is associated with free trisomy 21 per se and not with differential selection based on sex (Hassold et al. 1983). Molecular studies demonstrated a highly increased sex ratio (3.50) among paternal meiotic errors in contrast to paternal mitotic errors and maternal errors (Petersen et al. 1993), and that the excess of male probands was specific to MII errors (Savage et al. 1998). This suggested that they may be mechanisms of paternal nondisjunction in which the extra chromosome 21 preferentially segregates with the Y chromosome, as already hypothesized from cytogenetic studies (Hassold et al. 1984). Finally, an excess of Y-bearing sperm disomic for chromosome 21 was observed in semen samples from healthy volunteers, providing conclusive evidence that the excess of males in trisomy 21 is attributable at least in part to paternal nondisjunction (Griffin et al. 1996).

6

Suggested Risk Factors for Nondisjunction other than Maternal Age

6.1

Apolipoprotein E Allele e4

Many factors were suggested as risk factors for nondisjunction in the past, but only in a few recent studies has the origin of nondisjunction been determined by DNA analysis. No risk factor has been determined in young mothers who, despite their low individual risk, give birth to the majority of DS children. Results of a study (Avramopoulos et al. 1996) showed an increase in the frequency of apoE allele e4 as a risk factor for meiosis II nondisjunction of chromosome 21 in young mothers. The increased frequency of apoE allele e4 in a subgroup of young mothers with DS children supports their increased risk of Alzheimer disease (AD) as allele e4 is a genetic susceptibility factor for AD (Schupf et al. 1994). A biological role of apoE in the oocyte and the pathological role of the allele e4 in chromosomal nondisjunction remain to be investigated. ApoE is produced in most organs, including the ovary, in which it might be involved in chromosomal segregation. Meiosis II spindle dysfunction could be explained by both the isoform-specific binding of apoE to microtubule-associated proteins, as seen in AD, and by the possible interference of apoE with microtubule stability and function. This hypothesis would allow prediction of a higher number of meiosis II errors of DS in populations with a higher frequency of apoE allele e4.

Because many factors are probably involved in chromosomal nondisjunction, and meiosis II errors are responsible for about 25% of DS cases of

maternal origin, differences in the proportion of meiosis II errors could be expected in different populations. This could be addressed in population-based studies of DS with DNA polymorphism analysis. It would be of interest to investigate apoE genotypes of other trisomies and their parents to see if the increased frequency of apoE allele e4 is restricted to young mothers of trisomy 21 probands or if it is a common risk factor for chromosomal nondisjunction. It is possible that trisomic fetuses with the e4 allele often do not survive to term, influencing in this manner the observed apoE genotypes of parents with newborn DS babies. This would predict a higher frequency of allele e4 in DS fetuses compared to newborn babies, or a higher number of e4 homozygotes in fetuses.

6.2

Reduced Ovarian Complement

The question of whether the chronological age of the mother or the physiological age of the ovary is more important has both biological and clinical relevance. If oocyte depletion with advancing age is the basis of the maternal-age effect, as was suggested by Warburton (1989), then women who have a reduced number of oocytes for other reasons might have an increased risk for a conception with trisomy 21. In this regard, it is not unusual for women to have ovarian surgery for a variety of conditions, and, because of associated infertility, a number of these women become candidates for in vitro fertilization (IVF) (Khalifa et al. 1992). Brook et al. (1984) were the first to suggest that a unilateral oophorectomy (ULO) could be a risk factor for DS. Observing that mice with a ULO had premature onset of cycle irregularity and an early rise in aneuploidy, they concluded that the risk for DS is determined by the distance in time from the menopause (physiological age) rather than the chronological age of the mother and that the number of follicles limits the reproductive life span. Similarly, Warburton (1989) suggested that if oocyte depletion is the major factor in age-related nondisjunction in humans, women who have had a trisomic conception at a young age might exhibit signs of early oocyte depletion, such as premature menopause. Since the original report in mice, other studies have reported evidence of decreased reproductive fitness in women with a ULO. Many changes seen in these women are also seen with advancing maternal age in women with two ovaries. For example, higher FSH (Khalifa et al. 1992; Backer et al. 1999), lower estrogen (Lass et al. 1997), and shorter menstrual cycles (Hardy and Kuh 1999), hallmarks of advanced maternal age, have also been associated with ULO. These similarities suggest that age-related changes may be a matter of physiological rather than chronological age.

Although many reports describe the reproductive status of women with ULO, two recent reports have indicated that women who have had an aneuploid conceptus exhibit elevated serum FSH. Freeman et al. (2000) suggest

that the physiological status of the ovary—and, more specifically, the number of follicles—may be a key factor in maternal meiotic nondisjunction. The limited oocyte pool hypothesis proposed by Warburton (1989) states that an oocyte in a suboptimal state of development could become the dominant follicle because of the small number of oocytes available in older women. An oocyte of this type might be more likely to exhibit chromosome nondisjunction. The challenge still remains to determine exactly how a depleted oocyte pool could lead to recruitment of an oocyte destined to undergo nondisjunction when meiosis resumes.

6.3

Polymorphisms in Genes Involved in Folate Metabolism

Folic acid is essential for the *de novo* synthesis of nucleotide precursors for normal DNA synthesis and is also essential for normal cellular methylation reactions. Chronic folate/methyl deficiency *in vivo* and *in vitro* has been associated with abnormal DNA methylation (Balaghi and Wagner 1993; Pogribny et al. 1995; Fowler et al. 1998; Jacob et al. 1998), DNA strand breaks (Blount et al. 1997; Pogribny et al. 1997; Duthie 1999), altered chromosome recombination (Knuutila et al. 1978; MacGregor et al. 1997), and aberrant chromosome segregation (Libbus et al. 1990; Leyton et al. 1995; Chen et al. 1998; Titenko-Holland et al. 1998; Xu et al. 1999). On the basis of this evidence, James et al. (1999) suggested the possibility that gene-nutrient interactions associated with abnormal folate metabolism and DNA hypomethylation might increase the risk of chromosome nondisjunction. The MTHFR (methylenetetrahydrofolate reductase) gene catalyzes the synthesis of 5-methyltetrahydrofolate, the methyl donor for the B₁₂-dependent remethylation of homocysteine to methionine via the methionine synthase reaction. The reduction in enzyme activity associated with the 677C→T MTHFR polymorphism raises the dietary requirement for folic acid to maintain normal remethylation of homocysteine to methionine (Bailey and Gregory 1999).

Methionine synthase reductase (MTRR) is a related flavoprotein that maintains the methionine synthase enzyme in an active state for the remethylation of homocysteine to methionine. Because of the importance of the methionine synthase reaction in maintaining normal folate metabolism and DNA methylation, Hobbs et al. (2000) hypothesized that MTRR 66A→G polymorphism could be another maternal risk factor for Down syndrome. Preliminary studies had implicated the MTHFR 677C→T polymorphism (James et al. 1999) and excessive smoking (Yang et al. 1999) as maternal risk factors for DS. Interestingly, low folate status has been associated with each of these potential risk factors (Piyathilake et al. 1994; Lewis et al. 1998; James et al. 1999). Lymphocytes from women consuming a controlled folate-deficient diet were found to have significantly increased frequency of kinetochore-

positive micronuclei, which are surrogate markers for abnormal chromosome segregation (Titenko-Holland et al. 1998). Folate supplementation after the folate-depletion phase in this metabolic study was associated with a significant decrease in these centromeric fragments.

Taken together, these studies support the possibility that multifactorial gene-environment interactions that compromise maternal folate status may promote meiotic nondisjunction and the risk of a DS conception. Hobbs et al. (2000) suggested that the ability to analyze the MTHFR and MTRR genotypes in terms of specific metabolic biomarkers—such as plasma homocysteine, folate, and/or B₁₂ levels—would increase the power to detect a significant impact on DS risk. A compromise in the methionine synthase reaction caused by genetic and/or dietary factors could promote abnormal chromosome segregation by an indirect effect on oocyte DNA methylation patterns and higher-order chromatin structure. The secondary structure of pericentromeric heterochromatin, at repetitive satellite sequences, is involved in protein-DNA binding and in cohesion between sister chromatids (Renaud and Gasser 1997; Clarke 1998; Cobb et al. 1999).

6.4

Presenilin-1 Polymorphism

The presenilins are membrane proteins but their exact biological functions are still unknown (Cruts et al. 1996; Hutton and Hardy 1997). A recent study used immunofluorescence and electron microscopy to localize the presenilins to the nuclear membrane, kinetochores, and centrosomes, suggesting a role in chromosome organization and segregation (Li et al. 1997). Presenilin-1 (PS-1) is expressed in most regions of the human brain and in several peripheral tissues, including high protein levels in testis and lung (Sherrington et al. 1995; Suzuki et al. 1996). The predicted structure of the protein contains multiple transmembrane domains, implying that the protein is an integral membrane protein (Sherrington et al. 1995) engaged in intercellular interaction with APP (Dewji and Singer 1997; Xia et al. 1997) and with a further proposed role in the control of apoptosis (Guo et al. 1997). Rare forms of early onset, autosomal-dominant AD are caused by mutations in the APP gene on chromosome 21 (Goate et al. 1991), the presenilin-1 (PS-1) gene on chromosome 14 (Sherrington et al. 1995), and the presenilin-2 (PS-2) gene on chromosome 1 (Levy-Lahad et al. 1995).

Due to the suggested function of the presenilins in chromosome segregation, Petersen et al. (2000) decided to examine the distribution of PS-1 alleles and genotypes in cases of trisomy 21 of known parental and meiotic origin and their parents from a population-based study of DS. This study reported an increased frequency of allele 1 in the PS-1 gene in mothers with an MII error and in mothers carrying apoE allele e4. The frequency of allele 1 in mothers with a meiosis II error (70.8%) was significantly higher than in

mothers with a meiosis I error (52.7%). The frequency of allele 1 in mothers with a meiosis II error was also significantly higher than in fathers of maternal II errors. The association of susceptibility polymorphisms with complex diseases found its first example in apoE and AD (Roses 1997). These findings suggested that the PS-1 intronic polymorphism could be involved in chromosome nondisjunction due to the subcellular localization of the presenilins through an influence on the expression level of PS-1 or due to linkage disequilibrium with biologically relevant variability elsewhere in or outside the PS-1 gene (Theuns et al. 2000). The exact biological role of PS-1 in the human ovary and testis and a hypothetical allele-specific function on chromosomal segregation remain to be investigated.

6.5

Maternal Cigarette Smoking and Oral Contraceptive Use

Despite many years of research to identify risk factors associated with DS, only one factor has been well established, advanced maternal age. The search for specific environmental risk factors has yielded few definitive results (Hassold and Jacobs 1984; Kline et al. 1985). The effect of smoking has been examined and several studies have reported a nonsignificant negative association between maternal smoking around the time of conception and the risk for DS (Kline et al. 1983, 1993; Hook and Cross 1985; Shiono et al. 1986). Several investigations have suggested that this may be the result of selective prenatal loss of trisomic conceptuses among women who smoke (Kline et al. 1993; Hook and Cross 1988). However, others have concluded that there is no association between DS and periconceptional smoking (Christianson and Torfs 1988; Van den Eeden et al. 1990; Cuckle et al. 1990; Källén 1997).

It is important that previous studies on the effects of maternal smoking and many other factors on the risk for DS have pooled all trisomic individuals together regardless of the parent of origin or the timing of the chromosome error. Yang et al. (1999) were the first to categorize DS cases by the parent of origin and the timing of the chromosome error to increase the power to identify important environmental or maternal health-related risk factors. The findings of this study demonstrated an increased risk for DS in the offspring of women who smoke cigarettes around the time of conception. This effect is confined to maternal MII (MMII) cases among women younger than 35 years of age. These results may be due to (1) small sample size, (2) recall bias, (3) differential intrauterine survival, or (4) a true effect of smoking on the meiotic process. Sample size will remain an issue, particularly with the less frequent MMII cases, until additional subjects can be enrolled to confirm these findings. Recall bias is not a likely explanation for the differences in risk between MMI and MMII cases among younger mothers. Differential intrauterine survival of MMII cases among mothers who smoke around time of conception also seems unlikely. If this were the correct explanation and

MI fetuses were more sensitive to factors in the intrauterine environment, an increased ratio of MMI to MMII fetuses among spontaneous abortions compared with livebirths would have been expected.

There is little direct evidence of an effect of smoking on meiosis. However, it is well documented that maternal smoking delays conception, reduces fecundity, increases the risk for spontaneous or early fetal death, and causes early menopause (Baird and Wilcox 1985; Landesman-Dwyer and Emanuel 1979; Werler et al. 1990; Baron et al. 1990). With respect to the effect of oral contraceptive (OC) use on the risk for DS, studies have produced conflicting results, but in general, no association has been established. Some have suggested that women who take oral contraceptives during the months just before conception or who have OC breakthrough pregnancies have an increased risk of having a baby with DS (Lejeune and Prieur 1979; Harlap and Eldor 1980; Mikkelsen 1981). Others have found no evidence of an increased risk for DS among the offspring of mothers who use OC around the time of conception (Janerich et al. 1976; Ericson et al. 1983; Lammer and Cordero 1986; Källén 1989). A delay in conception after discontinuing OC use has been reported, but whether this is due to anovulation or an abnormality within the ovum is not known (Bracken et al. 1990).

Pregnancy loss before clinical recognition of the pregnancy may be incorrectly interpreted as an inability to conceive and, in this regard, it is well known that the major cause of early fetal loss is aneuploidy (Hassold et al. 1993). The risk for DS in the offspring of women who smoked and used OC around the time of conception was significantly increased, and this risk was confined to MMII cases (Yang et al. 1999). Because OC use among smokers carries an increased risk for thromboembolism, it is possible that the microvasculature of the ovary could be compromised. Blockage of small vessels by thromboembolic events would impair the delivery of oxygen to the follicles and their developing oocytes. This in turn might affect the spindle or some other component of the meiotic mechanism such as the chromosome stabilizing chiasmata. The fact that maternal smoking ($\pm$ OC) increased the risk for an MMII error and not for an MMI error may shed light on the processes leading to nondisjunction (Yang et al. 1999).

7

Summarizing Risk Factors

An interesting finding is that several of the suggested risk factors were found associated with meiosis II events in young mothers. One way to identify maternal age-related risk factors associated with nondisjunction is to examine maternal health factors and environmental exposures in mothers who had a maternally derived error. If a factor is identified as increasing the risk for nondisjunction, it may be possible to characterize that risk in the

context of aging. For example, as a woman ages, the number of follicles maturing to the preovulatory stage at each menstrual cycle decreases; only one of those follicles progresses to ovulation. Thus, a decrease in the number of maturing follicles is postulated to decrease the probability that one of them will be at the precise stage necessary for optimal response to follicle stimulation hormone (FSH), the trigger for ovulation (Sherman et al. 2005). Another approach to determine if aberrant genes may be involved in human nondisjunction is to examine the association of consanguinity and trisomy 21. If such an association was found, this could provide evidence for a genetic effect for nondisjunction. Alfi et al. (1980) postulated the existence of a gene that increases the risk for nondisjunction. Alternatively, they suggested that increased rates of consanguinity among parents would be correlated with those in grandparents and therefore, an autosomal recessive gene may be postulated to be involved in meiotic nondisjunction in the homozygous parents. Since that time, reports have found no evidence for an association between consanguinity and human nondisjunction (Sherman et al. 2005). Lastly, differences in the prevalence of DS among different racial groups may provide indirect evidence for genetic factors involved in human nondisjunction. However, such studies are difficult to conduct and to interpret. Differences (or similarities) may reflect the maternal age distribution of the population, completeness of ascertainment among infants and/or prenatal diagnoses, accuracy of diagnosis, cultural preference and/or access to selective prenatal termination of pregnancies with trisomic fetuses, and as yet unidentified environmental factors. Other factors such as alcohol, maternal irradiation, fertility drugs, spermicides, parity and low economic status have been implicated but not confirmed (Sherman et al. 2005).

8

Concluding Remarks

Chromosomal nondisjunction poses a considerable risk for the human species. We have reviewed the genetic studies of the recent years regarding origin and mechanisms of nondisjunction in human trisomy 21. There is altered meiotic recombination (number and position of chiasmata), and this is so far the only molecular correlate with nondisjunction.

Studies from model organisms make it clear that a wide variety of genetic and environmental disturbances can affect aneuploidy levels. Large population-based studies that separate individuals with DS by type error are in progress and will aid in the identification of risk factors that have remain elusive. The coming years will probably bring us more insight into the complex mechanisms underlying chromosomal segregation, but it is doubtful that the frequency of gametic nondisjunction can be reduced.

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Meiosis in *Arabidopsis thaliana*: Recombination, Chromosome Organization and Meiotic Progression

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Abstract Arabidopsis has emerged as an excellent system for meiosis research due to the development of powerful cytological, genetical and molecular methodologies combined with particular biological features of plant meiosis. This chapter reviews these developments and highlights their impact on the investigation of meiotic recombination. We focus specifically on meiotic recombination, but it is important to bear in mind that research on somatic DNA repair and recombination in Arabidopsis is a highly active field. Indeed, many aspects of somatic and meiotic recombination overlap and several genes are involved in both processes.

An overview of the features of meiotic recombination in Arabidopsis is obtainable from cytogenetic analysis of chiasma frequency and distribution and also from high-throughput genetic linkage analysis using molecular markers. Recombination parameters derived from these different approaches are in excellent agreement. Biotic and abiotic factors have been shown to influence recombination frequency in Arabidopsis. Forward and reverse genetic procedures have played prominent roles in the functional analysis of meiosis in Arabidopsis. A large and growing number of genes that are directly or indirectly involved in the meiotic recombination process and its regulation have been identified and analysed. These range from genes involved in the initiation of recombination through programmed double strand breaks, through the processing of these events as recombination intermediates to their final emergence as mature reciprocal crossover events (COs). Although the molecular events of recombination are largely conserved across eukaryotes, comparisons with other model species indicate some interesting though poorly understood differences. In common with other systems that have been analysed it is clear that the regulation of meiotic recombination in Arabidopsis involves a carefully programmed progressive selection of a relatively small number of recombination initiations to become COs. Evidence is also presented for the existence of more than one pathway to COs in Arabidopsis as in some other eukaryote systems. Finally, meiosis is increasingly being viewed as a highly complex series of interrelated events that are necessarily closely coordinated. In this context there is an emerging understanding, from Arabidopsis and other species, that recombination proceeds in the context of complex alterations in chromatin organization and that meiotic progression is dependent on the correct sequence and timing of these events.

Abbreviations

AE	Axial element
BrdU	Bromodeoxyuridine
cM/Mb	CentiMorgans per Megabase
CO	Crossover

Col	Columbia ecotypes
dHj	Double Holliday junction
DSB	Double strand break
FISH	Fluorescent in situ hybridization
GFP	Green fluorescent protein
LE	Lateral element
Ler	Landsberg erecta ecotype
rDNA	Ribosomal DNA
PMC	Pollen mother cell
RFP	Red fluorescent protein
RI	Recombination intermediate
RNAi	RNA interference
SC	Synaptonemal complex
T-DNA	DNA tagged by T-plasmid segments from <i>Agrobacterium tumefaciens</i>
TF	Transverse filament

1

Arabidopsis as a System for the Study of Meiosis

Our understanding of the mechanisms involved in meiosis and their genetic regulation have advanced rapidly in recent years due to the application of molecular genetics, in combination with genetical analysis and cytology (Roeder 1997; Zickler and Kleckner 1999). Much of this effort has been focussed on the simple unicellular fungi *Saccharomyces cerevisiae* (budding yeast) and *Schizosaccharomyces pombe* (fission yeast). However, parallel studies have been conducted in a range of other eukaryotic model species such as the filamentous fungus *Sordaria macrospora*, the invertebrate animals *Caenorhabditis elegans* and *Drosophila melanogaster*, the mouse *Mus musculus* and now, following genome sequencing and the development of a range of molecular tools, the flowering plant *Arabidopsis thaliana*. Other important plant models for meiosis research include maize, *Zea mays*, and rice, *Oryza sativa* (Hamant et al. 2006). Part of the motivation for these parallel studies is to establish the similarities and differences of meiotic processes and their control between different eukaryote groups (Loidl 2000), which should further our understanding of the fundamentals of meiosis. Certain interesting differences have already emerged from this comparative approach, such as the finding that formation of the synaptonemal complex (SC), a conserved meiotic feature, is dependent on prior programmed double-strand DNA breaks in some organisms, but not in others (reviewed by Loidl 2000). However, on the whole the similarities are more striking than the differences, testifying to the remarkable evolutionary conservation of meiosis and in particular of DNA repair and recombination processes.

What then are the particular advantages of including *Arabidopsis* among this group of model organisms for the investigation of meiosis, apart from

the obvious one of widening the phylogenetic base of the comparative approach to include Angiosperms? The answer to this question lies in the fact that methodologies developed in *Arabidopsis* in combination with particular biological features of plant meiosis provide an excellent system for investigating this important process.

1.1

Developments in Cytogenetic and Molecular Approaches Combine to make *Arabidopsis* Ideal for Meiosis Research

Flowering plants have figured importantly in earlier “classical” studies of meiosis because the large genomes, and hence large chromosomes, of many species and their superior cytology, made them ideal for cytogenetic analysis. Paradoxically, *Arabidopsis* chromosomes are notoriously small, though they are not unique or exceptional among plants in this respect, and until recently cytogenetic analysis of this species was regarded as challenging. Nevertheless, the development of improved preparative techniques has meant that the cytogenetic analysis of *Arabidopsis* meiosis is now routine (Mercier et al. 2001a; Ross et al. 1996, 1997).

The improvements in cytogenetics have occurred alongside the continued development and refinement of *Arabidopsis* as the model system for plant molecular genetics and genomics. These developments have enabled the use of forward and reverse genetic approaches to identify and characterize a large and growing number of *Arabidopsis* meiotic genes (Table 1), and mutations of genes of interest have been isolated by screening T-DNA or transposon transformed populations or pools of lines in order to conduct

Table 1 List of the *Arabidopsis* meiotic genes mentioned in this chapter, and their proposed functions

Gene name	Homologue	Function	Refs.
<i>ASY1</i>	<i>Hop1</i>	Axis-associated; required for synapsis	Armstrong et al. 2002
<i>ZYP1</i>	–	SC transverse filament	Higgins et al. 2005
<i>AtCAP1</i>	<i>SMC2</i>	Condensin	Siddiqui et al. 2003
<i>AtCAP2</i>	<i>SMC2</i>	Condensin	Siddiqui et al. 2003
<i>AtSMC1</i>	<i>SMC1</i>	Cohesin	Lam et al. 2005b
<i>AtSMC3</i>	<i>SMC3</i>	Cohesin	Lam et al. 2005b
<i>AtSCC3</i>	<i>SCC3</i>	Cohesin	Sanchez-Moran (unpublished)
<i>SYN1</i>	<i>Rec8</i>	Cohesin	Bai et al. 1999
<i>ASK1</i>	<i>SKP1</i>	SYN1 localization; nuclear reorganization	Zhao et al. 2006

Table 1 (continued)

Gene name	Homologue	Function	Refs.
<i>AtSPO11-1</i>	<i>SPO11</i>	Programmed double strand breaks (DSB)	Grelon et al. 2001
<i>AtSPO11-2</i>	<i>SPO11</i>	Programmed double strand breaks	Stacey et al. 2006
<i>AtRAD50</i>	<i>RAD50</i>	DSB processing; strand resection	Bleuyard et al. 2004
<i>AtMRE11</i>	<i>MRE11</i>	DSB processing; strand resection	Puizina et al. 2004
<i>ATCOM1</i>	<i>COM1/SAE1</i>	DSB processing; strand resection	Schlogelhofer (personal comm.)
<i>AtNBS1</i>	<i>NBS1</i>	DSB processing; strand resection	Armstrong and West (unpublished)
<i>AtRAD51</i>	<i>RAD51</i>	Strand invasion; D-loop formation	Li et al. 2004
<i>AtDMC1</i>	<i>DMC1</i>	Strand invasion; D-loop formation	Couteau et al. 1999
<i>AtRAD51C</i>	<i>RAD51C (m)</i>	<i>RAD51/DMC1</i> accessory factor	Li et al. 2005
<i>AtXRCC3</i>	<i>XRCC3</i>	<i>RAD51/DMC1</i> accessory factor	Bleuyard and White 2004
<i>AtBRCA2</i>	<i>BRCA2</i>	<i>RAD51/DMC1</i> accessory factor	Siaud et al. 2004
<i>AtMND1</i>	<i>MND1</i>	<i>DMC1</i> accessory factor	Kerzendorfer et al. 2006
<i>MPA1</i>	-	Required for early steps in recombination	Sanchez-Moran et al. 2004
<i>MEI1</i>	-	Required for DNA replication processes	Grelon et al. 2003
<i>AtCDC45</i>	<i>CDC45</i>	Required for DNA replication processes	Stevens et al. 2004
<i>At MSH4</i>	<i>MSH4</i>	Stabilization of dHJs; directing some to COs	Higgins et al. 2004
<i>AtMSH5</i>	<i>MSH5</i>	Stabilization of dHJs; directing some to COs	Higgins (unpublished)
<i>AtMER3/RCK</i>	<i>MER3</i>	Stabilization of dHJs; directing some to COs	Mercier et al. 2005
<i>PTD</i>	-	Promotion of COs; dHj resolution?	Wijeratne et al. 2006
<i>AtMSH2</i>	<i>MSH2</i>	CO suppression	Emmanuel et al. 2006
<i>AtMUS81</i>	<i>MUS81</i>	Minor promotion of COs	Higgins (unpublished)
<i>AtMLH1</i>	<i>MLH1</i>	Drive <i>MSH4/5</i> -selected RIs towards COs	Jackson et al. 2006
<i>AtMLH3</i>	<i>MLH3</i>	Drive <i>MSH4/5</i> -selected RIs towards COs	Jackson et al. 2006
<i>SWI1</i>	-	Candidate meiotic initiation regulator	Mercier et al. 2003
<i>AML1-5</i>	<i>AML1-5</i>	Regulator of meiosis?	Kaur et al. 2006
<i>QUARTET</i>	-	Required for microspore separation	Copenhaver et al. 2000

The homologues listed are from budding yeast or fission yeast, except where indicated otherwise. A few are novel genes with no known homologues in other species *m* mammalian homologues

functional analysis (Caryl et al. 2003). Most recently, proteomics has provided an alternative approach to identifying meiotic genes that may prove to be especially applicable to identifying genes showing little or no conservation at the primary sequence level (Sanchez-Moran et al. 2005). Since it is known that certain genes may be up-regulated during meiosis, or have meiosis-specific splice variants (Caryl et al. 2003), expression profiling using microarray technology provides another possible route to gene identification. Directed gene disruption by homologous recombination (as in yeasts and mouse) is not possible at present in *Arabidopsis*, but gene silencing or down-regulation procedures, such as antisense RNA and RNA interference (RNAi) are available and have been successfully applied to the functional analysis of *Arabidopsis* meiotic genes in cases where functional mutational knockouts were not available (Higgins et al. 2005; Siaud et al. 2004). Another approach that is actively being pursued to identify genes that exhibit a meiotic phenotype is the production of gain-of-function mutants by insertion of transcriptional enhancers (activation tagging) (Clara Conicella, personal communication).

The molecular cloning of meiotic genes has in turn played a significant part in the development and/or application of molecular cytogenetic techniques, such as immunocytology, GFP-fusion and fluorescent in situ hybridization (FISH). Together these have greatly extended the scope for the analysis of normal and defective meiosis. Furthermore, the application of electron microscopy and immunogold localization of meiotic proteins have led to greatly improved resolution of chromosome synapsis and recombination related structures.

Genetic linkage analysis based on visible marker genes has a long history in *Arabidopsis* but suffers in that it is rather laborious. However, more recently the introduction of molecular markers, including high-throughput genotyping methods for handling single nucleotide polymorphisms (Drouaud et al. 2006) has led to great improvements in genetic mapping and recombination analysis. Furthermore, the exploitation of the *quartet* mutation in *Arabidopsis*, in which the four pollen grains resulting from a single meiosis fail to separate, has enabled the development of tetrad analysis systems that are capable of providing detailed information on diverse recombination parameters, including interference and, potentially, gene conversion rates (Copenhaver et al. 2002). Initially, these analyses relied on analysing PCR-based DNA markers in the four progeny that result from controlled pollinations with unseparated pollen tetrads (Copenhaver et al. 2000), but more recently a much more powerful and direct visual system has been developed that uses expression of marker proteins in pollen. Another relatively high-throughput recombination assay has also been developed based on the use of lines carrying seed-expressed fluorescent (GFP and RFP) colour markers (Melamed-Bessudo et al. 2005).

1.2

Inherent Properties of Meiosis in Arabidopsis Make it Well Suited for Analysis

In addition to technical developments, some inherent biological factors have also contributed to Arabidopsis becoming a valuable system for meiosis research. The synchrony of meiotic progression among the 50 or so pollen mother cells (PMCs) in each Arabidopsis anther locule has permitted the development of a simple method for determining meiotic time courses for wild-type and mutant plants based on bromodeoxyuridine (BrdU)-labelling of nuclei in meiotic S-phase, sampling at different time points and detection of label at different meiotic stages by anti-BrdU antibody (Armstrong et al. 2003). This procedure has allowed the measurement of delayed meiotic progression in mutants and also enabled the accurate timing of the appearance and disappearance of meiotic proteins detected immunocytologically (Fig. 1). Female meiosis in Angiosperm plants occurs in embryo-sac mother cells (EMCs) within the protected environment of ovules and generally occurs slightly later in plant development than male meiosis. Arabidopsis has 40–50 ovules per gynoecium, each containing a single EMC, and methods have been developed for observing meiosis in these cells and following the subsequent development of the female gametophyte (Armstrong and Jones 2001; Schneitz et al. 1995).

Plants appear to have one particular advantage over other eukaryote groups for meiotic analysis, which is that meiotic checkpoints that are activated in certain mutants lead to delayed meiotic progression but not meiotic arrest (e.g. Jackson et al. 2006). This means that meiosis in most Arabidopsis

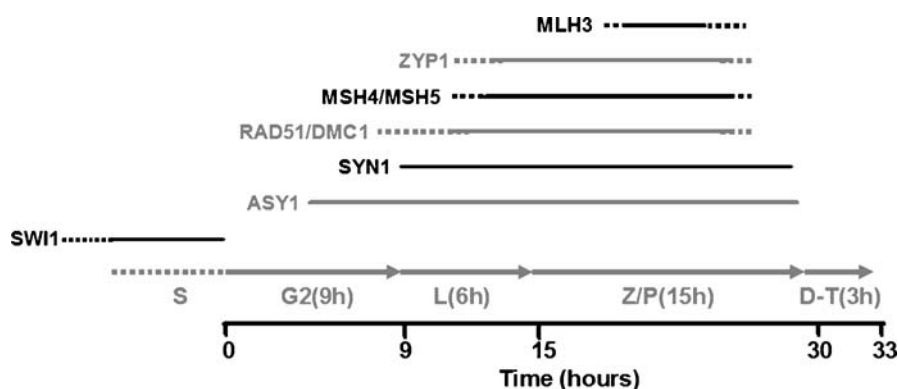


Fig. 1 Time course of meiosis in Arabidopsis. A 2 h pulse of BrdU was delivered to meiocytes via the transpiration stream, followed by sampling of anthers at 4 h intervals and detection of labelled cells by anti-BrdU antibody. The expression profiles of meiotic proteins were determined in dual immunolocalization experiments with anti-BrdU and antibodies to meiotic proteins

mutants proceeds to completion, albeit with some delay, and thus aspects of the mutant phenotype are not confounded with abnormalities resulting from meiotic arrest and the onset of apoptosis. Furthermore, because meiosis is not arrested at an early stage in mutants their recombination characteristics can be assessed by either cytological means (chiasmata at metaphase I) or genetically among surviving progeny.

Finally, *Arabidopsis* is a close relative of the crop Brassicas (*Brassica oleracea*, *B. napus* etc.) within the family Cruciferae. In addition to providing a potential outlet for transfer of knowledge from *Arabidopsis* to crop plants, the close affinity and high level of synteny between *Arabidopsis* and *Brassica* permits the functional analysis of meiotic genes in the cytologically more amenable *Brassica* system (Armstrong et al. 2002). These and other advantages of the *Arabidopsis* system have meant that within a few years it has been possible to adopt a fully integrated approach to the analysis of meiosis and meiotic recombination with input from genetics, cytology and molecular biology (Jones et al. 2003).

2

Recombination in *Arabidopsis*; an Overview

2.1

Cytological and Genetic Methods

for Assessing Meiotic Recombination are in Good Agreement

Arabidopsis has only five pairs of chromosomes and hence the recombination of physically unlinked genes, on different chromosomes, through independent assortment is relatively limited but is still an important contribution to the overall level of recombination. The amount of recombination of linked genes through crossing over and chiasma formation has been assessed by a variety of genetical and cytological approaches that give reassuringly consistent values for genome-wide and chromosome-specific recombination in *Arabidopsis*. Chiasma frequency in plants is best assessed at metaphase I where, despite the disadvantage of maximum condensation, there is no risk of confusing chiasmata and relational twists. The chromosomes of *Arabidopsis* can be individually distinguished for the purpose of chiasma counting following FISH with probes for 45S and 5S rDNA. Analysis of a large sample of pollen mother cells from the commonly employed accession Columbia (Col) yielded a mean chiasma frequency of 9.10 per cell (Sanchez-Moran et al. 2002), which agrees closely with estimates from genetical recombination analysis. For example, (Copenhaver et al. 1998) detected an average of 8.91 reciprocal recombination events per pollen mother cell over 78% of the genome from an analysis of meiotic tetrads, which is also equivalent to the overall recombination level measured in recombinant inbred lines (Lister and Dean 1993).

To reinforce this encouraging correspondence between cytological and genetical data, it has recently been shown that the MutL homologues MLH1 and MLH3, that are known to mark the sites of reciprocal recombination events in other species, immunolocalize to ~ 9 – 10 foci on *Arabidopsis* pachytene bivalents (Jackson et al. 2006). Turning attention to individual chromosomes, chromosome 4, one of the two shorter (acrocentric) chromosomes has been particularly well-investigated both genetically and cytologically and once again there is a close agreement between the different methods of assessment. Cytologically, chromosome 4 has a mean of 1.62 chiasmata in the Col accession whereas genetical estimates of CO frequencies from Col or from Col $\times$ Ler crosses give estimates of 1.5 (Copenhaver et al. 1998), 1.52 (Lister and Dean 1993) and 1.6 (Drouaud et al. 2006).

More detailed analyses of recombination rates across chromosome regions and whole chromosomes of *Arabidopsis* are now beginning to be undertaken with the aim of understanding the basis of recombination variation and its regulation. Drouaud et al. (2006) carried out an analysis of recombination rates across chromosome 4 of *Arabidopsis* based on 71 single nucleotide polymorphisms covering the entire chromosome, on 702 F2 plants. They found that genetic recombination rates were very variable across the chromosome, ranging from 0 cM/Mb near the centromere to 20 cM/Mb on the short arm next to the NOR region, with a chromosome average of 4.6 cM/Mb. Further analysis showed that recombination rates correlated negatively with the G + C content and positively with the density of single repeats and the CpG ratio, but not with genes, pseudogenes, transposable elements or dispersed repeats. A detailed analysis of several regions with high CO rates revealed hotspots of recombination contained in small fragments of a few kilobases, which by and large explained the variation of CO rates across the chromosome.

By analysing inter-CO distances on informative two-CO chromatids they also found evidence of non-independence of CO positions, from which they concluded that CO location on chromosome 4 is affected by interference. Using a different approach, Copenhaver et al. (1998) exploited the *quartet* mutation of *Arabidopsis* (see above) to determine parameters of male meiotic recombination and found that CO distribution across the *Arabidopsis* genome was affected by interference. The number of double COs in adjacent regions (33) was 65% lower than the number expected from genetic map data on the basis of CO independence (93). Subsequently it was proposed that CO distribution in male meiosis of *Arabidopsis* best fits a model where the majority (85%) of COs show interference, but a minority of COs, possibly originating from a different recombination pathway, are interference-free and sprinkled randomly across the genome (Copenhaver et al. 2002). A rider to this hypothesis proposes that chromosomes 2 and 4 have a larger proportion of interference-dependent COs than chromosomes 1, 3 and 5 and that this may relate to their bearing nucleolus-organizing regions on their short arms that could serve the function of pairing regions (Lam et al. 2005a).

2.2

Recombination Frequency is Influenced by Biotic and Abiotic Factors

Genome-wide and chromosome-specific recombination frequency is known to be subject to variation in many eukaryotic organisms and several factors are known to be involved including genotype, environment and epigenetic factors, especially sex, or more correctly gender (Korol and Iliadi 1994). Significant chiasma frequency variation has been detected among different accessions of *Arabidopsis* grown under constant environmental conditions, ranging between 9.24 and 7.90 chiasmata per cell (Sanchez-Moran et al. 2002). Further analysis confirms that the chiasma frequencies of individual chromosome pairs are, broadly speaking, proportional to chromosome size. Chromosome 1, the longest chromosome has a mean chiasma frequency of 1.98 in the Col accession, whereas the two shortest (acrocentric) chromosomes, 2 and 4, have 1.68 and 1.62 chiasmata, respectively. However, the chiasma frequencies of individual chromosome pairs do not vary consistently across different accessions. Interestingly the most variable chromosome across accessions is chromosome 4, while the least variable is the other acrocentric chromosome (2) (Sanchez-Moran et al. 2002).

Recombination frequency, assessed genetically or cytologically, has been shown to differ significantly between sexes in many species (Lorch 2005), but there is no consistency in the direction or magnitude of the difference. Female meiosis was examined cytologically in EMCs of *Arabidopsis* from the Ws accession (Armstrong and Jones 2001). The mean chiasma frequency of EMCs was estimated to be 8.5 per cell, which is distinctly lower than the corresponding value for male meiosis in PMCs of the same accession (9.24) (Sanchez-Moran et al. 2002). This observation is consistent with genetical recombination differences reported by Vizir and Korol (1990) and Barth et al. (2001)

Environmental effects on recombination have been extensively documented in a wide range of species (Nilsson 1994). Such effects are also known to operate in *Arabidopsis*. Barth et al. (2001) developed a high-throughput system for genome-wide measurement of recombination in *Arabidopsis*, based on transgenic markers. Using this system they demonstrated, as proof of principle, significant effects of temperature and phosphate treatments on recombination in *Arabidopsis*, but these effects were region-dependent.

3

Understanding the Molecular Basis of Meiotic Recombination in *Arabidopsis*

Ultimately, a full understanding of meiotic recombination and its regulation in *Arabidopsis*, as in other model species, is dependent on gene identification and functional analysis. Initially, candidate meiotic genes were identified by screening collections of T-DNA or transposon tagged lines for reduced fer-

tility or sterility, and this approach proved to be relatively successful (Caryl et al. 2003). Several of these genes were found to share homology with meiotic genes from other organisms e.g. *ASY1* and *HOP1*; *SYN1* and *REC8*, whereas others appeared to be novel genes with no known homologues (e.g. *SWI1*). Following the sequencing of the Arabidopsis genome it became possible to search for Arabidopsis homologues of known meiotic genes from other species, especially budding yeast. This latter approach has proved to be especially useful for gene identification, but is particularly applicable to genes that show relatively high degrees of conservation, including genes involved in the catalytic steps of DNA recombination. Some other candidate Arabidopsis genes with less direct, more structural or supporting, recombination functions, including cohesins and condensins have also been identified by this approach. On the other hand, genes that encode structural components of the SC are known to show very little conservation at the primary sequence level, though the proteins show similar features of secondary structure, and other approaches are required for the identification of these genes (Bogdanov et al. 2003). A list of the Arabidopsis meiotic genes mentioned in this chapter is given in Table 1.

3.1

Early Recombination Events; Formation and Processing of Double Strand Breaks

Meiotic recombination in eukaryotes results from the processing of programmed double strand breaks (DSBs) in chromosomal DNA and these DSBs are regarded as the initiating event of meiotic recombination. However, it is likely that DSB formation is preceded by other, as yet unidentified processes, perhaps involving chromatin reorganization, that determine their locations. In all organisms so far investigated, meiotic DSBs are catalysed by the Spo11 protein, a member of a family of type II-like topoisomerases that possesses transesterase activity (Bergerat et al. 1997; Keeney et al. 1997). Three potential homologues of *SPO11* have been identified in Arabidopsis, two of which (*AtSPO11-1* and *AtSPO11-2*) are now known to be required in meiosis (Grelon et al. 2001). Both genes are highly expressed in reproductive tissues, but in common with many other meiotic genes they are also expressed at lower levels in other tissues (Grelon et al. 2001; Hartung and Puchta 2000). Immunolocalization studies with an antibody to the *AtSPO11-1* protein found that the protein is only very transiently present on chromosome axes at the leptotene stage since foci were only rarely detected over a small proportion of the wild-type nuclei examined (E. Sanchez-Moran, unpublished). However, Arabidopsis mutants that are unable to process DSBs, such as *Atcom1* accumulate DSBs and exhibit abundant *AtSPO11-1* foci. *AtCOM1* is the Arabidopsis homologue of the yeast meiotic gene *COM1/SAE2* that is known to be required for the processing of DSBs (Prinz et al. 1997). The *com1/sae2* mutants in yeast closely resemble the phenotype of *rad50S* mutants. The phenotype of the *At-*

com1 mutant in *Arabidopsis* is consistent with the gene performing the same function as in yeast (P. Schlogelhofer, personal communication).

It is known that the histone variant H2AX is rapidly phosphorylated (γ H2AX) in a large region (50 kb) surrounding DSBs (Mahadevaiah et al. 2001), thus providing an immunocytological indicator of DSBs that is not dependent on accumulation of DSBs in mutants. In *Arabidopsis* γ H2AX first appears during leptotene and accumulates through zygotene giving a diffuse immunofluorescent signal. By pachytene the signal reduces to give a few discrete foci that approximately correspond in number to the number of reciprocal CO events (E. Sanchez-Moran, unpublished).

Two mutant alleles of *AtSPO11-1* were initially identified from T-DNA transformed and EMS mutagenized Versailles populations (*Atspo11-1-1* and *Atspo11-1-2*, respectively) (Grelon et al. 2001). The *Atspo11-1-1* mutant, thought to be a null mutant on the basis of the T-DNA insertion site, exhibited a chromosome synapsis defect during prophase I but, unexpectedly, it was not completely recombination defective. Cytological examination of metaphase I revealed that univalents predominated but a few bivalents were formed (chiasma frequency 7% of wild-type) and a low frequency of recombination was detected in genetical tests (Grelon et al. 2001). The corresponding *Drosophila* and *C. elegans* mutants show no recombination and it was therefore thought initially that some DSBs can be formed by an alternative route in *Arabidopsis*, such as one of the other *AtSPO11* homologues or environmental DNA damage. However, recent analysis of a third mutant allele of *AtSPO11-1* (*Atspo11-1-3*), from the SALK T-DNA transformant population, indicated that this mutant was completely recombination defective. No chiasmate bivalents were observed in 150 cells at metaphase I, and other immunological indicators of recombination intermediates, such as γ H2AX and AtRAD51 foci, were also absent (E. Sanchez-Moran and J.L. Santos, unpublished). This finding therefore raises the possibility that the previously described *Atspo11-1-1* mutant is not a null allele; possibly it may be able to express a truncated protein, with some limited activity, by translation from an alternative ATG site downstream of the T-DNA insertion. Immunolocalization of the *Arabidopsis* SC transverse filament protein ZYP1 confirms that synapsis does not occur in *Atspo11-1-3*.

The cytogenetical meiotic phenotype of *Atspo11-2* (Stacey et al. 2006) is very similar to that seen in the *Atspo11-1-3* null mutant, that is, complete absence of bivalents at metaphase I. Accordingly *AtSPO11-1* and *AtSPO11-2* are thought to function non-redundantly in the same recombination pathway during meiosis, which could indicate that the proteins act cooperatively, perhaps as a complex although there is as yet no evidence that they form a dimer. It is interesting to note, in this context, that double heterozygotes *Atspo11-1-3/+*; *Atspo11-2/+* show mild to severe sterility phenotype, indicative of a genetic interaction that has been termed non-allelic non-complementation (Stacey et al. 2006).

SPO11-induced DSBs are resected to yield 3' single-stranded tails that are required for the next step in the meiotic recombination process. In budding yeast the MRX complex, comprising *MRE11*, *RAD50* and *XRS2/NBS1* (members of the *RAD52* epistasis group), is required both for the formation of SPO11-induced DSBs and the processing by resection of these ends. Mutants of these genes in budding yeast are deficient in meiotic recombination and SC assembly with a concomitant severe reduction in spore viability. The similarity of the phenotypes observed in several eukaryotes defective for homologues of the MRX complex components suggest that meiotic functions of the MRX complex are conserved among eukaryotes, including *Arabidopsis* (Bleuyard et al. 2004). It has been commented that since mutation in many members of the *RAD52* epistasis group result in embryonic lethality in mammals, much of our knowledge on the meiotic roles of these proteins in "higher" eukaryotes comes from invertebrates and plants. *Arabidopsis* homologues of *RAD50*, *MRE11* and *NBS1* have been identified in *Arabidopsis* (Daoudal-Cotterell et al. 2002). *Arabidopsis* mutants of these genes (*Atrad50*, *Atmre11*, *Atnbs1*) have been analysed and shown to have severe meiotic phenotypes, including failure of chromosome synapsis, defective recombination and extensive chromosome fragmentation in post-prophase I stages (Bleuyard et al. 2004; Puizina et al. 2004). In both *Atrad50* and *Atmre11* the chromosome fragmentation phenotype was rescued in double mutants with *Atspo11-1*, indicating that the fragmentation is AtSPO11-1-dependent and represents unrepaired DSBs (Bleuyard et al. 2004; Puizina et al. 2004). Analysis of *Atmre11* reveals a significant difference to the budding yeast, *Coprinus cinereus* and *C. elegans* homologues in that the *Arabidopsis* protein does not appear to be required for DSB formation.

3.2

Strand Invasion and Joint-Molecule Formation

The yeast homologues of bacterial RecA protein, Rad51 and Dmc1, have been shown to be essential for inter-homologue recombination and bivalent formation. In meiosis these proteins work together to catalyse invasion of the homologous non-sister DNA molecule by single-stranded resected 3' ends from double-strand breaks. Both genes are highly conserved in eukaryotes, including *Arabidopsis*, but their functions appear to have diverged to some extent. In budding yeast both proteins are required for meiotic recombination, but Rad51 has additional functions in mitotic recombinational DNA repair. In contrast, the loss of Rad51 is lethal in both mouse and chicken cells, indicating that in vertebrates it is required for the maintenance of genome integrity, possibly reflecting their greater genome complexity and different chromatin structure. This conclusion may, however, be premature because of the limited range of eukaryotes in which Rad51 function has been investigated. Thus, analysis of AtRAD51 function in *Arabidopsis* shows that

the protein is dispensable for vegetative development, though required for meiosis. This indicates that the functions of this protein may vary widely in different species, independently of such factors as genome complexity. A T-DNA insertional knockout mutant in *Arabidopsis* (*Atrad51*) showed normal plant growth and flower development, but was completely sterile (Li et al. 2004). Cytological examination revealed that meiosis was disrupted in this mutant, with failure of chromosome synapsis and recombination, and extensive AtSPO11-dependent chromosome fragmentation.

Although Rad51 and Dmc1 are believed to work cooperatively to catalyse strand invasion, the single mutant phenotypes are distinct in *Arabidopsis*, as in mouse and perhaps all multicellular eukaryotes. Both mutants are defective in chromosome synapsis and recombination, but whereas *Atrad51* exhibits severe chromosome fragmentation, *Atdmc1* has ten unfragmented univalent chromosomes at comparable stages (Couteau et al. 1999). Thus, *Atrad51* mutants are unable to repair DSBs efficiently, while *Atdmc1* mutants are able to repair from the intact duplexes of sister chromatids (Siaud et al. 2004). Five *RAD51* paralogues, also known as *RAD51*-like genes, (*Rad 51B*, *C*, *D*, *Xrcc2* and *Xrcc3*) have been identified in mammals and birds, where they form complexes that perform essential roles in DNA repair.

Homologues of these five *RAD51* paralogues have been identified in the *Arabidopsis* genome and the functions of two of them (*AtRAD51C* and *AtXRCC3*) have been investigated by the analysis of T-DNA insertional mutants (Bleuyard and White 2004; Li et al. 2005). The phenotypes of these mutants are very similar, both to each other and to *Atrad51*, involving failure of synapsis, a recombination defect and extensive AtSPO11-dependent chromosome fragmentation. It was initially concluded that *Atxrcc3* had normal synapsis, despite having a recombination defect and extensive chromosome fragmentation (Bleuyard and White 2004). Subsequent work has now established that synapsis is in fact defective in this mutant (C. White personal communication).

This suggests that all three proteins are involved in the same key process of promoting the inter-homologue strand invasion that is required for homologue pairing and synapsis, recombination and, coincidentally, the repair of DSBs. However, the fact that each of these three mutants has severe defects indicates that the three genes have distinct functions. It has been suggested therefore that *AtRAD51C* and *AtXRCC3* form a complex that facilitates the loading and/or activity of *AtRAD51* during meiotic prophase. Evidently a number of accessory factors are operating to facilitate or modulate the complex molecular events that are involved in homologue recognition and the initial steps of chromosome synapsis and recombination.

A further example of such a factor is provided by *AtBRCA2*, the *Arabidopsis* homologue of the *BRCA2* gene associated with hereditary breast cancer in humans and the maintenance of genome integrity in embryonic mice. *Arabidopsis* has two *BRCA2*-like genes and RNAi silencing of these genes at

meiosis generated a sterility phenotype that was associated with severe meiotic disruption including chromosome fragmentation and non-homologous chromosome fusions (Siaud et al. 2004). This phenotype is strongly reminiscent of that obtained upon RNAi silencing of the *AtRAD51* gene in an *Atdmc1* mutant background. Inactivation of *AtRAD51*, *AtDMC1* and *AtBRCA2* gave a similar phenotype to *AtBRCA2* inactivation or inactivation of both *AtRAD51* and *AtDMC1*, suggesting that the proteins act in the same pathway and may indeed interact physically, as has been shown in a yeast two-hybrid assay (Siaud et al. 2004).

Similarly, it has recently been established that the Arabidopsis homologue of *MND1* (*AtMND1*) (Kerzendorfer et al. 2006), that in other systems works as a heterodimer with Hop2 (AHP2 in Arabidopsis) is needed for AtDMC1-dependent meiotic recombination and repair between homologous chromosomes, but not for AtRAD51-dependent recombination and repair between sister chromatids (P. Schlogelhofer, personal communication). *Atmnd1* mutants are deficient in chromosome pairing and synapsis and also show AtSPO11-dependent chromosome fragmentation. A novel meiotic gene *MPA1*, which encodes a puromycin-sensitive aminopeptidase, also appears to have an important accessory role during the early recombination pathway (Sanchez-Moran et al. 2004). In a majority (> 90%) of *mpa1* meiocytes AtRAD51 accumulates in the cytoplasm rather than forming chromatin-associated foci. Meiotic proteins such as AtMSH4 and AtMLH3, which are involved in later steps in the recombination pathway, also fail to localize to the chromatin.

Almost all the genes listed above that are required for the processing of DSBs at the strand resection step or the strand invasion step have in common that loss of function, by mutation or RNAi, leads to AtSPO11-dependent chromosome fragmentation. However, it is possible that not all chromosome fragmentation mutant phenotypes originate from this cause. Two cases have been reported from Arabidopsis where chromosome fragmentation has been shown to be AtSPO11-independent and therefore unlikely to originate from unrepaired DSBs. The Arabidopsis *MEI1* gene was first described as a gene involved in male meiosis, encoding a protein showing homology with a human acrosin-trypsin inhibitor (He et al. 1996). Grelon et al. (2003) obtained a new mutant allele and showed that the gene mutated actually encodes a much longer protein that contains five BRCT domains and is similar to proteins involved in the response to DNA damage and replication blocks during the mitotic cell cycle. The *Atspo11-mei1* double mutant had a high level of chromosome damage, thus revealing that the chromosome fragmentation could not be due to unrepaired AtSPO11-generated DSBs. Grelon et al. (2003) considered it very likely that MEI1 is involved in some DNA processes linked to replication, such as the resolution of stalled replication forks. RNAi-induced silencing of an Arabidopsis *CDC45* homologue resulted in a very similar phenotype to *mei1*, including AtSPO11-independent chromosome fragmentation

(Stevens et al. 2004) from which they also deduced a defect in meiotic DNA replication during the meiotic S-phase.

A caveat to the conclusions reached in these studies is that the *AtSPO11* allele used in the studies was *Atspo11-1-1*, which as previously mentioned may not be a null mutation.

3.3

Two CO Pathways in Arabidopsis

Evidence from budding yeast indicates the existence of a selection process by which a subset of recombination precursors are directed along a pathway that will end in COs, while all others go primarily to non-COs. It has been proposed that this decision occurs at an early stage, at or before the appearance of stable strand exchanges (Boerner et al. 2004). This transition is accompanied by the loading of proteins that have important, though imperfectly understood, roles in the stabilization of recombination intermediates and in their direction towards a CO fate. In budding yeast a group of proteins, the so called ZMM proteins (Zip1, Zip2, Zip3, Msh5 and Mer3) and their corresponding genes are implicated in this decision and, simultaneously, in the imposition of CO interference (Boerner et al. 2004). Mutants for all these genes and all double mutant combinations have virtually identical phenotypes, suggesting that they act at the same point in the same pathway, presumably as components of a protein complex. In Arabidopsis homologues of two of these proteins, AtMSH5 and AtMER3 (RCK), as well as AtMSH4 that works together with AtMSH5 as a heterodimer, are known to be involved in the direction of recombination intermediates towards COs (Chen et al. 2005; Higgins et al. 2004; Mercier et al. 2005). Since the mutant phenotypes of these three genes are almost identical it seems likely that the proteins act cooperatively in the same pathway as part of a complex, just as in budding yeast. For this reason we present the detailed functional analysis of only one of these genes, *AtMSH4*, which was the first to be completed.

Expression of *AtMSH4*, the Arabidopsis homologue of *MSH4*, could only be detected in floral tissues, consistent with a role in reproduction and immunofluorescence studies. This indicated that its expression is limited to early meiotic prophase I, preceding the synapsis of homologous chromosomes. A T-DNA insertional mutant (*Atmsh4*) exhibited normal vegetative growth but a severe reduction in fertility, consistent with a meiotic defect that was confirmed by cytological analysis of meiosis. A time-course study, based on BrdU labelling of nuclei in S-phase, showed that prophase I chromosome synapsis is delayed by about 8 h and electron microscopical analysis of prophase I spreads found that synapsis may be incomplete in *Atmsh4*. Metaphase I chiasma frequency of *Atmsh4* is greatly reduced to about 15% of wild-type, leading to univalence and non-disjunction.

A statistical analysis, comparing numerical distributions of chiasmata to the Poisson distribution, revealed that these residual chiasmata are randomly distributed among cells and chromosomes. Boerner et al. (2004) have estimated that approximately 15% of COs in budding yeast arise independently of the main class I (ZMM) pathway, and suggest that these could be products of the non-CO branch of the recombination pathway. In addition, de los Santos et al. (2003) indicated that class II COs appear to be more prominent between shorter chromosomes of budding yeast, suggesting that recombination responds to chromosome size by modulating the relative numbers of class I and class II COs. Copenhaver et al. (2002) have argued that the genetically determined CO distribution in *Arabidopsis* is also compatible with the existence of two pathways for crossing over.

The *quartet* mutation of *Arabidopsis* was exploited to analyse CO distribution in meiotic tetrads. They found that the fit to a theoretical “chi-square” distribution, which has been widely applied to modelling CO interference, was substantially improved by assuming an additional set of COs sprinkled at random among those distributed as per chi-square. Based on this analysis, they argued for the existence for two pathways for crossing over in *Arabidopsis*, as in budding yeast but unlike *Drosophila* and *C. elegans*, only one of which exhibits interference. Furthermore they estimated that the proportion of COs without interference (p) was generally close to 0.20, though it occasionally fell below 0.10. The finding that the residual chiasmata in the *Atmsh4* mutant constitute 15.7% (1.55/9.86) of the wild-type value is remarkably consistent with this prediction. It is, of course, an inference that the *AtMSH4*-independent chiasmata in the *Atmsh4* mutant are also present in wild-type as part of its normal complement of chiasmata. However, the analysis conducted by Copenhaver et al. (2002) supports this contention.

Taken together, the various sources of evidence indicate strongly that *Arabidopsis* possesses two CO pathways, exactly as predicted for budding yeast. However, the proposed absence of interference between the residual COs in *Atmsh4* is an inference from the statistical properties of chiasma distribution in the mutant and has not been directly determined by more exact tests. On the other hand, Mercier et al. (2005) provide unambiguous data showing that the residual COs in *Atmer3* are interference-free at one set of adjacent intervals. A novel gene *PARTING DANCERS* (*PTD*) has also been implicated in the interference-dependent pathway. In common with the ZMM class of mutants, COs in a *ptd* mutant exhibit a random distribution. However, it is proposed that *PTD* acts at later stage in the recombination pathway than the ZMM proteins, possibly at the stage of dHJ resolution (Wijeratne et al. 2006).

Whereas Msh4/Msh5 actively promote meiotic CO recombination, it is known from studies in budding yeast and mammals that Msh2 as a consequence of its MMR activity, can inhibit recombination between divergent DNA sequences (Chambers et al. 1996; Chen and Jinks-Robertson 1999; Elliott and Jasin 2001). A recent study to analyse the role of the *Arabidopsis*

homologue found that AtMSH2 has a broad range of anti-recombination effects both in somatic cells and during meiosis. In particular, meiotic recombination between homologous chromosomes derived from different ecotypes showed a 40% increase compared to wild-type in the absence of AtMSH2. This is claimed to be the first report of Msh2-mediated repression of meiotic recombination between homologues, as opposed to homoeologues (Emmanuel et al. 2006).

In 2003, de los Santos et al. proposed that budding yeast possesses an alternative pathway for CO formation, based on the processing of non-dHj intermediates. This pathway requires the activity of an endonuclease, Mus 81, which acts as a heterodimer with another protein, Mms4 (de los Santos et al. 2003; Hollingsworth and Brill 2004). This realization led to the proposal that there are two classes of COs in budding yeast, originating from two distinct biochemical pathways. Most COs belong to class I, exhibit CO interference and are dependent on Msh4/Msh5. Class II COs are a minority, do not exhibit interference and are dependent upon Mus81/Mms4 (de los Santos et al. 2003). However, evidence from other studies indicates that the contribution of these two classes to total COs can vary a great deal between different organisms. So, for example, in the fission yeast, *Schizosaccharomyces pombe*, all COs appear to be class II events. On the other hand, in *C. elegans* *msh4* and *msh5* mutants completely eliminate crossing-over, indicating that all COs in this organism belong to class I. Msh4 and Msh5 are known to be required for the proper execution of meiosis in the mouse since *msh4* and *msh5* knockout mice are sterile (de Vries et al. 1999; Kneitz et al. 2000). In contrast, *mus81* knockout mice are fully fertile (McPherson et al. 2004) suggesting that possibly only the class I recombination pathway is active, as in *C. elegans*. However, a minor contribution of the class II pathway to meiotic recombination in the mouse cannot be excluded on this evidence.

Arabidopsis possesses two apparent homologues of *MUS81*, only one of which appears to be expressed and so it is of interest to determine whether the activity of this gene accounts for the ~ 15% interference-free residual COs in *Atmsh4*. A T-DNA insertional mutation (*Atmus81*) has no detectable effect on fertility, as in the mouse, and in addition pollen mother cell chiasma frequency is not significantly reduced compared to wild-type. However, preliminary evidence from the *Atmsh4/Atmus81* double mutant indicates that the *AtMUS81* gene accounts for ~ 0.40 chiasmata per cell, about one third of the *Atmsh4* residual chiasmata (J. Higgins, unpublished observations). This implies that one or more further genes may be responsible for the remaining class II COs. The proposed existence of a minority sub-class of chiasmata that do not exhibit interference has implications for current estimates of recombination frequency in *Arabidopsis* based on chiasma analysis. We infer that ~ 80% of cells in wild-type *Arabidopsis* have at least one (1–7) interference-free class II CO. A proportion of these is therefore likely to occur nearby other COs and, as such, will be difficult or impossible to detect cytologically.

This may explain the small but significant deficit of metaphase I chiasmata compared to genetical estimates of recombination frequency in *Arabidopsis* (Sanchez-Moran et al. 2002).

3.4

The Evolution of Recombination Intermediates into Mature COs; the Role of *AtMLH1/3*

The eukaryotic homologues of the *Escherichia coli* MutL mismatch repair (MMR) protein play important roles in maintaining genome stability during both mitosis and meiosis (Hoffmann and Borts 2004; Kolodner and Marsischky 1999). Studies in budding yeast have identified four MutL homologues that form functionally distinct heterodimers. Two of these, Mlh1/Pms1 and Mlh1/Mlh2 are proposed to have roles in the correction of different classes of DNA mismatch, whereas the Mlh1/Mlh3 heterodimer appears to play an important role in promoting meiotic COs in budding yeast (Wang et al. 1999) and in mouse (Baker et al. 1996; Lipkin et al. 2002). It has been known for some time that the *Arabidopsis* genome contains *AtMLH1* and *AtMLH3* (originally called *AtMLHx*) homologues. Recently these genes have been cloned and subjected to functional analysis (Jackson et al. 2006). Immunolocalization studies have established that the proteins appear as foci on synapsed chromosomes at mid- to late prophase I. Their numbers gradually increase until there are approximately 9–10 foci per nucleus at pachytene, coinciding closely with the number of chiasmata recorded cytologically and the number of genetically detected COs. Dual immunolocalization with anti-*AtMLH1* and anti-*AtMLH3* antibodies showed that the proteins co-localize at foci that are presumed to be the sites of reciprocal recombination events (COs).

Further analysis of *AtMLH3* was made possible by the identification of two T-DNA insertional mutants, both of which appeared to be null mutants based on absence of the corresponding RNA transcripts and proteins. Vegetative growth of both mutants was indistinguishable from wild-type but both revealed reduced fertility phenotypes consistent with a meiotic defect. Cytological, including immunocytological, examination revealed that the early events of prophase I, including pairing and synapsis of homologues, proceeded as normal and this was accompanied by normal loading of early and mid-prophase recombination proteins *AtRAD51* and *AtMSH4*. However, from diplotene onwards it became clear that fewer chiasmata than normal were formed, leading to the production of some univalents and consequent mis-segregation of chromosomes. This reduction in chiasma frequency is accompanied by a substantial delay of ~ 25 h in meiotic progression. Analysis of chiasma frequencies at metaphase I found that mean chiasma frequency, in both mutants, was reduced to about 35% of wild-type but with an unusually wide range of cell chiasma frequencies (0–9). Statistical analysis showed that the distribution of cell chiasma frequencies in the mutants agrees closely with

the binomial distribution. This finding is wholly consistent with the proposition that in wild-type the AtMLH3 protein drives a sub-set of recombination intermediates (dHJs) towards COs, whereas in the absence of this protein two alternative outcomes are possible, COs (35%) or non-COs (65%).

A complication in interpreting the residual chiasmata seen in *Atmlh3* is that they could include the ~ 1.5 chiasmata per cell that are attributable to the class II pathway. However, since these are thought to be interference-free, a proportion of these chiasmata could occur too close to others to be resolved cytologically.

4

Meiotic Progression, Chromosome Organization and Recombination

The initiation of meiosis in budding yeast is known to depend on the nutritional status of the medium and on the genotype of diploid cells. Furthermore, key regulatory genes have been identified that are involved in the induction of meiosis and which trigger the activation of a cascade of early meiotic genes that are required for the initiation of recombination and chromosome synapsis. Little is known concerning the genetic control of meiotic induction in plants though similar or related processes are presumed to occur. However the Arabidopsis *SWI1* gene is a candidate meiotic initiation regulator although it shows no similarity to known genes or genomic sequences from other species (Chambers et al. 1996; Chen and Jinks-Robertson 1999; Elliott and Jasin 2001; Mercier et al. 2001b, 2003; Motamayor et al. 2000). One indication of this comes from immunolocalization experiments coupled with BrdU labelling, showing that *SWI1* is expressed very early, exclusively in meiotic G1 and S-phase. Secondly, a presumptively null mutant allele, *swi1-2* shows a range of defects including total absence of homologue synapsis and precocious loss of sister-chromatid cohesion. In addition, immunolocalization studies established that axial elements do not assemble and recombination is probably not initiated. Thus, although the cells retain some superficial resemblance to meiocytes, it is probable that this mutant fails to initiate meiosis properly. At the very least these observations indicate that *SWI1* has a pivotal role in early meiosis.

In the *ameiotic1-1* (*am1-1*) mutant of *Zea mays* meiotic divisions are replaced by mitotic divisions, indicating a role for this gene in meiotic initiation. Indeed it is reported that the Am1 protein shows partial sequence similarity to Arabidopsis SWI1 protein, strongly suggesting that these two proteins are functional homologues despite some important differences in their mutant phenotypes (Hamant et al. 2006). The Arabidopsis-MEI2-like genes comprise a five-member gene family, related to the MEI2 gene which is a master regulator of meiosis in the fission yeast *S. pombe*. Based on the expression patterns of these genes and functional analysis based on RNAi

lines and insertional mutants showing a range of meiotic defects, it has been suggested that these genes may be involved in the regulation of meiosis in *Arabidopsis*, indicating a possible conservation of function across plants and fungi (Kaur et al. 2006)

The normal progression of meiosis in wild-type *Arabidopsis* has been established in time-course experiments that involve labelling cells in meiotic S-phase with BrdU, sampling at intervals and detecting the progress of labelled cells through meiosis using anti-BrdU antibody (Armstrong et al. 2003). By this means the total duration of male meiosis in *Arabidopsis*, from the end of the S-phase to the tetrad stage, occupies 33 h at 18.5 °C. The greater part of this time is taken up by a distinct G2 period (9 h) and an extended prophase I (21.3 h). The remaining meiotic division stages are completed very rapidly, within 3 h.

It has been remarked that plants are unusual in that meiotic mutants that are defective in various critical steps of chromosome synapsis and recombination generally manage to progress through meiosis to completion, albeit often with unbalanced chromosome sets in the resulting microspores (Couteau et al. 1999). This has led to the supposition that plants lack the robust meiotic checkpoints that result in arrest and failure to complete meiosis in other groups of organisms. However, the determination of meiotic time courses shows that meiotic progression is subject to varying delay in *Arabidopsis* meiotic mutants. For example, *Atmsh4* experiences a delay of 8 h in meiotic progression while in *Atmlh3* meiosis completion is delayed by a massive 25 h (Higgins et al. 2004; Jackson et al. 2006). These cases indicate the existence of a surveillance mechanism in *Arabidopsis*, similar to that in budding yeast that maintains coordination between the recombination pathway and prophase I progression. Associated with this delayed progression, several mutants defective in some aspect of the recombination pathway also exhibit pleiotropic effects on chromosome organization. Most commonly, these mutants display mild chromosome condensation defects that are most apparent in prophase I (Bai et al. 1999; Higgins et al. 2004). This suggests that recombination processes and chromosome organization are linked mechanistically and both may be detected by the surveillance mechanism that senses problems with chromosomal processes and implements an appropriate response, in this case delayed meiotic progression.

There is only limited evidence that condensin and cohesin proteins impact directly on recombination processes in *Arabidopsis*, although this could reflect a paucity of studies. Siddiqui et al. (2003) found that mutations in the *Arabidopsis* condensin genes *AtCAP-E1* and *AtCAP-E2* (*SMC2* homologues) disrupted several processes in vegetative growth and development and also affected the segregation of homologous chromosomes during meiosis, which could in part be due to unspecified recombination defects. It is reported that the *Arabidopsis* genome encodes single copies of two other members of the cohesin complex *AtSMC1* and *AtSMC3* (Lam et al. 2005b). However, subse-

quent analysis has revealed a further member of this gene family (J. Jager and E. Sanchez-Moran, unpublished). It is annotated as a *SMC3* homologue, but the organization of conserved motifs in the predicted gene product suggests that it has some similarity to *SMC1 β* in mouse (Revenkova et al. 2001).

A T-DNA insertional mutant of this gene showed a significant chromatin condensation defect throughout meiosis. Associated with this, chiasma frequency was slightly reduced compared to wild-type and some non-homologous chromosome associations occurred. The *Arabidopsis ASK1* gene encodes a homolog of the human and yeast Skp1 proteins and is involved in several aspects of plant growth and development, including normal meiotic progression. The *ask1* mutant shows abnormalities of chromosome organization during meiosis, including defective chromosome condensation, synapsis and, ultimately, chromosome separation. Immunolocalization studies indicate that the prophase I distribution of SYN1 protein, a REC8 orthologue that is thought to possess cohesin function in *Arabidopsis*, is abnormal in the *ask1* mutant (Zhao et al. 2006). It is also pertinent to mention that a recent study in maize of an allelic series of *absence of first division* mutants (*AFD1* is a homologue of the meiotic cohesin *REC8*) revealed that the *AFD1* protein is required for the normal distribution of RAD51 foci in prophase I (Golubovskaya et al. 2006). These and other findings indicate that chromatin organization during meiosis is likely closely integrated with recombination events.

Probably the greatest chromosome organizational changes during meiosis occur in connection with the elaboration of the SC in prophase I. It is therefore of interest to examine the functions, especially in relation to recombination of structural components of the SC and also associated proteins. The axial elements (AEs), proteinaceous cores associated with each homologous chromosome, are elaborated during leptotene and later, following chromosome synapsis, they become the lateral elements (LEs) of the SC. Although protein components of the LEs have been identified and subjected to functional analysis in budding yeast and mouse, the equivalent LE components have not been identified in *Arabidopsis*. However, one of the earliest meiotic genes to be characterized in *Arabidopsis* was *ASY1*, an AE/LE-associated protein (Caryl et al. 2000).

By fluorescence immunolocalization the *ASY1* protein appears to colocalize with AEs/LEs, but immunogold electron microscopical localization shows that it is found in chromatin that is closely associated with these structures (Armstrong et al. 2002). A T-DNA insertional mutant (*asy1*) exhibits a strong recombination defect with chiasma frequency reduced to $\sim 15\%$ of the wild-type level (Sanchez-Moran et al. 2002). More recently it has been shown that AE formation appears to be normal in the mutant, as judged by immunolocalization of the cohesins AtSCC3 and AtSMC3. Synapsis, however, does not occur, based on lack of polymerization of the ZYP1 transverse filament protein (see below). Nevertheless DSB formation occurs with normal timing in the *asy1* mutant, as judged by phosphorylated γ H2AX immunolocalization

(E. Sanchez-Moran, unpublished). These and other related observations lead to the conclusion that ASY1 is required for the recruitment and transport of chromatin regions bearing DSBs to the AEs, where they are appropriately placed to engage in homologous recombination.

Structural protein components of the SC are notoriously difficult to identify by homology searches due to their low degree of conservation at the primary amino-acid sequence level despite sharing similar structural properties. The only Arabidopsis SC component that has been characterized to date is the transverse filament (TF) protein ZYP1, that was identified from comparisons of conserved secondary structure of TF proteins from budding yeast, *C. elegans*, *Drosophila* and mammals (Higgins et al. 2005). Immunolocalization detects ZYP1 foci at late leptotene, which lengthen until at pachytene fluorescent signals extend the entire lengths of fully synapsed bivalents covering the central region of the SC in between the flanking lateral elements. Interestingly, the ZYP1 protein is encoded by a closely linked pair of duplicated genes (*ZYP1A* and *ZYP1B*), a feature that effectively prevents the construction of a double knockout mutant by conventional means. After depletion of the ZYP1 protein by a combination of insertional mutagenesis and RNAi no ZYP1 protein can be detected and synapsis, but not alignment, of homologues is prevented. Recombination, as judged from numbers of AtMLH1 foci and chiasmata, is only slightly reduced (to about 80% of wild-type) in the absence of ZYP1 protein. However, COs occur between both homologous and non-homologous chromosomes, resulting in extensive multivalent formation and non-homologous bivalent associations. This suggests that in Arabidopsis this protein is directly or indirectly required to ensure the fidelity of meiotic chromosome association and recombination, which is a previously unreported function for TF protein. As in the case of some other recombination-defective Arabidopsis mutants, the *ZYP1*^{RNAi} lines exhibit a chromatin condensation defect that is most pronounced during prophase I and, associated with this, these lines suffer a pronounced delay of about 20 h in meiotic progression. These observations reinforce the previously noted coordination between recombination, chromosome organization and meiotic progression.

5

Crossover Control

It has long been recognized that the number and distribution of COs in eukaryotes is strictly regulated so that the number of COs per chromosome pair (bivalent) falls within a very narrow range. Generally there is an upper limit on their numbers while there is also a stringent control that ensures that each chromosome pair forms at least one CO, which is the basic requirement to ensure stable bivalent co-orientation and regular chromosome disjunction at

the first meiotic division (Jones 1984; Roeder 1997). In *Arabidopsis* the effect of these controls is strikingly obvious. The numbers of chiasmata per bivalent vary between one and three (very rarely four) and the numbers per cell are accordingly also constrained between eight and twelve (with rare exceptions). The mean chiasma frequencies of individual bivalents vary between about 1.6 and 2.0. How is all this achieved? We can begin to account for it in phenomenological terms by invoking the influences of such effects as positive CO interference and the obligate CO (Bishop and Zickler 2004; Jones and Franklin 2006), but these are descriptive terms and do not address the underlying mechanisms that are ultimately responsible.

Evidence from budding yeast and mammals indicates that the numbers of DSBs greatly exceed the numbers of COs that eventually result (Kauppi et al. 2004). This numerical reduction involves a progressive selection process that directs a subset of RIs towards a CO fate, while others are relegated to default pathways towards non-COs. Similar processes appear to be at work in *Arabidopsis* meiosis. Fluorescence immunolocalization indicates that the Spo11 protein is present at up to 100 foci during leptotene in *Arabidopsis* pollen mother cells, and this is therefore the best direct estimate that we have of DSB number (E. Sanchez-Moran, unpublished), although this maximum number is rarely observed presumably because of the rapid turnover of DSBs. However, supporting evidence comes from the regular observation of ~ 100 AtSPO11 foci in the *Atmre11* mutant that is unable to process DSBs. It appears that in wild-type meiosis most or all DSBs enter the early recombination pathway and become RIs since very similar numbers (~ 100) of foci are seen following immunolocalization of the early recombination proteins AtRAD51 and AtDMC1 (E. Sanchez-Moran, unpublished).

The available evidence from the analysis of *Atmsh4* mutant meiosis indicates that AtMSH4 protein is responsible, with its partner AtMSH5, for directing a subset of RIs towards COs (Higgins 2004). Moreover, biochemical studies using human MSH4 and MSH5 proteins have led to the proposal that the heterocomplex acts by stabilizing dHJs, and in some unknown way directing a proportion of them towards COs (Snowden et al. 2004). Thus it is rather surprising that the number of AtMSH4 foci seen over early prophase I nuclei is several-fold the eventual numbers of COs (Higgins 2004). It appears AtMSH4 is loaded onto all RIs, but is subject to an as yet undefined selective process that allows only a small subset to progress to form COs. The AtMLH3 protein has an important role in the final conversion of RIs into mature COs since in the absence of this protein there is a 60% reduction in COs. In wild-type meiosis an average of 9–10 AtMLH3 foci are observed over pachytene nuclei, which is consistent with their proposed role in directing the AtMSH4/5 preselected RIs to a CO fate. Understanding the processes involved in the selective progression of recombination intermediates towards crossovers and the simultaneous imposition of crossover control will be key targets for future research in *Arabidopsis*.

The ratio of COs to DSBs is not constant across all organisms. In budding yeast, extrapolating from genetic data, there appear to be two to three times as many DSBs as eventual COs (Fogel et al. 1981; Kauppi et al. 2004) whereas in mouse the ratio is close to 10 : 1; that is there are of the order of 250 cytologically detected early RIs, which reduce to about 23 COs (Moens et al. 2002). This ratio is similar to that seen in *Arabidopsis*. Intriguingly, it has recently been established from experiments in budding yeast that CO number shows strong homeostasis in the face of reduced numbers of DSBs (Martini et al. 2006), implying that DSB number is not the sole determinant of CO frequency and that the DSB to CO ratio is not fixed. It will be interesting to discover if this principle also applies in other eukaryotes, such as *Arabidopsis*, where the normal ratio of COs to DSBs is rather different.

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Modified Cell Cycle Regulation in Meiosis

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Abstract The study of meiosis regulation has always been carried out in parallel with mitotic cell cycle discoveries. The basic cell cycle machinery that regulates mitosis, based on fluctuations in the activity of cyclin-dependent kinases (CDKs), is responsible for the main transitions that occur during meiosis. However, the special characteristics of meiosis (e.g., the absence of an S-phase between meiosis I and meiosis II, a long prophase in which homologous recombination events occur, etc.) require specific regulation, and cells respond to this challenging situation in different ways. In some cases, mitotic regulators carry out the new functions or change their relative importance in a particular process, while in other cases novel meiosis-specific regulators emerge.

In this chapter, we shall analyze these special modifications, beginning with the specific signals that cells receive to exit the mitotic cell cycle and enter meiosis. We shall review how mitotic regulators adapt to the necessities of the meiotic program, paying particular attention to meiosis-specific factors whose functions are essential for meiosis to be completed successfully.

Abbreviations

APC/C	anaphase-promoting complex/cyclosome
CAK	CDK-activating kinase
CDC	cell division cycle protein
CDK	cyclin-dependent kinase
CSF	cytostatic factor
DSB	double-strand break
FEAR	CDC fourteen early anaphase release
GVBD	germinal vesicle breakdown
MAPK	mitogen-activated protein kinase
MEN	mitotic exit network
MCC	mitotic checkpoint complex
MPF	maturation/M-phase promoting factor
PKA	protein kinase A
PKB	protein kinase B
SAC	spindle assembly checkpoint
SC	synaptonemal complex

1

Meiosis Entry

Meiotic differentiation in higher eukaryotes and yeasts occurs with different aims. In animals, meiosis generates haploid gametes that are essential

for sexual reproduction, and in yeasts it leads to the formation of spores, which allow yeast survival under unfavorable environmental conditions. In both cases, the increase in genetic diversity, concomitant to the meiotic process, favors the adaptation of the species to a changing environment.

The signals that govern entry into meiosis differ in unicellular and multicellular organisms. While in unicellular organisms each cell has the ability to undergo meiosis, in most multicellular organisms it is possible to distinguish between germinal and somatic cells, and only germinal cells are able to undergo meiosis and produce gametes. It could be suggested that the main function of somatic cells is to confer protection to germ cells to guarantee the survival of species. The “meiosis entry” decision has been extensively studied in yeasts, and also in some animals, including mice, *Caenorhabditis elegans*, and *Drosophila*. In mammals, entry into meiosis occurs differently in male and female organisms. In females, during fetal development germ cells proceed to meiosis and oocytes arrest at the end of prophase I. Hormonal stimulation induces the resumption of meiosis, together with a second cell cycle arrest at metaphase II. Meiosis II is completed when the oocyte is fertilized. By contrast, in males meiosis is postnatal. Spermatogenesis starts prior to puberty and proceeds in waves throughout adult life (reviewed by Morelli and Cohen 2005). In *C. elegans* proliferating and meiotic cells are distributed along gonad arms: at the distal end there is a population of proliferating cells and, as germ cells move proximally, they enter meiotic prophase, meiotic divisions, and undergo gametogenesis. The proliferation–meiotic decision is controlled by two redundant mRNA regulatory pathways, GLD-1 and GLD-2, which promote meiotic development, and the GLP-1/Notch signaling pathway, which promotes proliferation through inhibition of the GLD-1 and GLD-2 pathways. Near the distal tip cells the GLP-1 signaling is active and thus cells proliferate. As cells move proximally, the GLP-1 signaling is inactive and thus cells enter meiosis (reviewed by Hansen and Schedl 2006). Similarly, in *Drosophila* an asymmetric division of germinal stem cells—perpendicular to the normal division plane—gives rise to a daughter cell that loses stem cell identity due to the lack of contact, and therefore signaling, with somatic proliferative cells. This daughter cell will become committed to meiotic fate (reviewed by Yamashita et al. 2005).

In low eukaryotes with sexual reproduction, entry into meiosis requires specific environmental signals. In yeasts, the nutrient supply regulates the switch from the mitotic cell cycle to the specialized meiotic program. Both fission and budding yeasts sense the lack of nutrients in the medium, especially nitrogen (reviewed by Kupiec et al. 1997, and Yamamoto 2004). In addition, meiosis initiation in budding yeast requires the presence of a nonfermentable carbon source, such as acetate, which can be metabolized through respiration, and the complete absence of glucose, which inhibits entry into meiosis (reviewed by Kupiec et al. 1997). In addition to environmental signals, budding and fission yeasts require the expression of both mating type genes in the same cell in order to undergo meiosis (*mat1-M* and *mat1-P* in *Schizosaccharomyces pombe*;

MATa and *MAT α* in *Saccharomyces cerevisiae*).¹ This ensures that meiosis only occurs in cells with two complete sets of chromosomes, and that haploid organisms are generated as a result of two consecutive segregations. In *S. pombe*, meiotic divisions are usually preceded by conjugation (zygotic meiosis) because fission yeast is a haploid organism. Also, fission yeast diploids can be maintained if zygotes are transferred to rich medium.² Diploid fission yeast cells initiate meiosis as soon as they sense the lack of nutrients (azygotic meiosis) (reviewed by Yamamoto 2004). This is not the case for budding yeast, since it is a diploid organism and conjugation occurs even in rich medium.

1.1

Meiosis Entry in *S. cerevisiae*

When internal and environmental conditions are met, two transcription factors essential for the switch from the mitotic to the meiotic program are upregulated: Ime1 in *S. cerevisiae*, and Ste11 in *S. pombe* (Fig. 1). During vegetative growth in budding yeast, G₁ cyclins (Clns) prevent entry into meiosis by inhibiting the transcription of *IME1* and its accumulation within the nucleus (Colomina et al. 1999). In the absence of nitrogen and of a fermentable carbon source, diploid cells arrest in G₁ and Cln protein levels decrease, allowing Ime1 activation and the transcription of genes essential for premeiotic DNA replication, homologous chromosome pairing, and recombination. The *IME1* promoter contains binding motifs for the Rme1 repressor, which inhibits the expression of *IME1* in haploid cells (reviewed by Honigberg and Purnapatre 2003). In diploid cells, the MATa/MAT α heterodimeric repressor reduces *RME1* expression, allowing the transcription of *IME1* (Mitchell and Herskowitz 1986). In addition, low levels of glucose, through the Ras-cAMP kinase pathway, and the presence of acetate, acting through specific regulatory elements of the *IME1* promoter, induce the expression of *IME1* (reviewed by Honigberg and Purnapatre 2003).

1.2

Meiosis Entry in *S. pombe*

In fission yeast, the expression of *ste11*⁺ is inhibited in rich medium because protein kinase A (PKA) phosphorylates and inhibits the transcription factor Rst2, which induces *ste11*⁺ transcription (Higuchi et al. 2002; Kunitomo et al. 2000). In addition, recent work has shown that Ste11 is inhibited by cyclin-dependent kinase (CDK) phosphorylation, which restrains its activation to the G₁ phase of the cell cycle (Kjaerulff et al. 2007). The heterodimer com-

¹ In fact, both *mat1-M* and *mat1-P* in *S. pombe*, as well as *MAT α* in *S. cerevisiae*, consist of two separate genes, each controlling different subfunctions.

² Only ~ 1% of early zygotes can thus be diverted to diploid mitosis; the majority remain committed to meiosis even on rich medium.

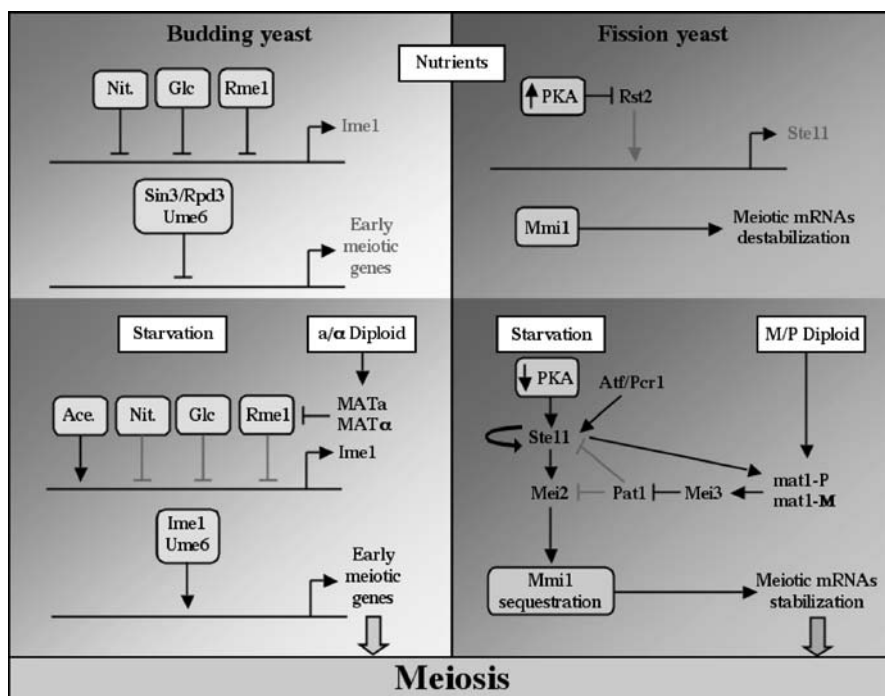


Fig. 1 Schematic representation of the main pathways controlling the entry into meiosis in budding and fission yeasts. In both yeasts, nutritional signals and mating type genes control the switch from mitosis to meiosis. Under growth conditions, the transcription of budding yeast *IME1* and fission yeast *ste11⁺* is inhibited. In addition, the fission yeast RNA binding Mmi1 protein destabilizes several meiosis-specific transcripts. In diploid budding yeast cells under starvation, *IME1* expression is upregulated and forms a complex with Ume6 that activates the transcription of early meiotic genes. In fission yeast, *ste11⁺* expression is induced in the absence of nitrogen. Ste11, in addition to its own transcription, controls the expression of *mei2⁺* and the mating type genes. In haploid cells the master regulator of meiosis Mei2 is inhibited by Pat1 phosphorylation. Pat1 also inhibits the nuclear localization of Ste11. In the presence of both mating type genes (i.e., diploid cells), Mei3 prevents the inhibitory phosphorylation of Mei2 by Pat1. The RNA-binding protein Mei2 sequesters Mmi1, thus leading to the stabilization of meiosis-specific transcripts. Ace: acetate; Glc: glucose; Nit: nitrogen. Black lines indicate participation in the process. Gray lines indicate inhibition of such participation

posed of Atf1/Gad7 and Pcr1 also participates in *ste11⁺* induction (Kano et al. 1996; Takeda et al. 1995; Watanabe and Yamamoto 1996). Ste11 is a HMG protein (high-mobility group protein) that binds to a T-rich motif, the TR box TTCTTTGTYY (Sugimoto et al. 1991), present in the promoters of genes whose expression is induced by Ste11, such as *ste11⁺* itself, the mating type genes, and the RNA binding protein and master regulator of meiosis Mei2. In haploid cells, Mei2 is phosphorylated and inactivated by the protein kinase

Pat1 (Watanabe et al. 1997). In addition, Pat1 phosphorylates Ste11, and phosphorylated Ste11 is excluded from the nucleus by binding to Rad24, a 14-3-3 protein (Kitamura et al. 2001; Li and McLeod 1996). Only when both mating type genes are expressed, that is in zygotes or in diploid cells, is Mei3 protein synthesized and, acting as a pseudosubstrate of Pat1, inhibits Mei2 phosphorylation (Li and McLeod 1996; McLeod and Beach 1988). Mei2 is essential for both premeiotic S-phase and meiosis I entry (Watanabe and Yamamoto 1994). During meiotic prophase, Mei2 binds to an RNA encoded by the gene *sme2⁺*, known as meiRNA. Mei2 forms a dot in the nucleus at the *sme2⁺* locus, indicating that the cell is ready for meiosis I entry. The binding of Mei2 to other RNAs is required for entry into premeiotic S-phase, since mutations that affect the RNA-binding ability of Mei2 inhibit premeiotic S-phase, but deletion of *sme2⁺* blocks meiosis progression only between premeiotic DNA replication and meiosis I (Watanabe and Yamamoto 1994). A mechanism by which Mei2 controls meiosis entry has recently been uncovered. Harigaya et al. (2006) have found that several meiosis-specific transcripts contain a region known as DSR (determinant of selective removal), to which the protein Mmi1 binds, promoting their degradation during vegetative growth. When meiosis is induced, Mei2 sequesters Mmi1 at the nuclear focus, allowing the expression of these meiosis-specific transcripts. This regulatory system controls the expression of an important number of meiotic regulators, such as the transcription factor Mei4, proteins involved in early meiotic events such as Rec8, Rec25, Bqt1, Ssm4, and Mug1, and the regulator of meiosis progression Spo5 (Harigaya et al. 2006).

2

Meiotic Expression Profiles

Genome-wide analyses of meiotic transcription have allowed the identification of novel meiotic genes whose expression is specifically enhanced during meiosis. The specific pattern of induction of these genes has anticipated their possible function, facilitating their analysis. In *S. pombe* and *S. cerevisiae*, recent deletion studies based on microarray data have uncovered novel genes with essential meiotic functions (Gegan et al. 2005; Martin-Castellanos et al. 2005; Rabitsch et al. 2001). Microarray analyses have also shed light on novel transcription factors, as well as the specific motifs to which these transcription factors bind.

2.1

Transcriptional Regulation During Meiosis in *S. cerevisiae*

In *S. cerevisiae*, seven groups of genes have been defined according to the time of their meiotic induction: very early, early (classified in early I, early II,

and early-late genes), middle, mid-late, and late genes (Chu et al. 1998; Primig et al. 2000). The analysis of meiotic promoters has unveiled regulatory elements targeted by specific transcription factors (reviewed by Vershon and Pierce 2000). In budding yeast, entry into sexual differentiation and the transcription of early genes require the transcription factor Ime1 (Fig. 1). The transcription factor Ume6 binds the “upstream repressor sequence”, URS1, present in the promoters of many early genes. During vegetative growth, the Ume6–Sin3–Rpd3 complex represses the transcription of early genes due to histone deacetylation of nucleosomes in the promoter region carried out by the Rpd3 histone deacetylase (Kadosh and Struhl 1997; Kasten et al. 1997). Under nutritional starvation and in the absence of glucose, the Sin3–Rpd3 complex is inactivated, and Ume6 associates with Ime1. Rim11 and Rim15 kinases, activated in the absence of PKA, stabilize the Ume6–Ime1 complex, and the binding of this complex to the URS1 sequences induces the expression of early genes. In late meiosis, Ime1 is phosphorylated by the meiosis-specific Ime2 kinase, leading to its degradation by the proteasome (Guttmann-Raviv et al. 2002).

Middle genes are involved in meiotic divisions and the initiation of spore formation. About 70% of the genes induced at this stage contain a “middle sporulation element”, MSE. The transcription factor Ndt80, which is essential for the activation of middle genes (Chu and Herskowitz 1998; Hepworth et al. 1998), binds MSE motifs, acting in competition with repressor factors such as Sum1 and Hst1 (Xie et al. 1999). One third of the middle genes do not contain MSE sequences. Some of them have URS1 sequences in their promoters, like the early genes. Also, Ndt80 could bind to noncanonical sequences or could be aided by an unknown cofactor.

Mid-late and late genes are involved in the formation of the spore wall. More than half of them contain MSE sequences, and are probably induced by Ndt80 and repressed by Sum1. In order to prevent their transcription during the middle stages of meiosis, some of these genes contain “negative regulatory elements” (NREs). For example, the *DIT1* and *DIT2* promoters present NREs, targeted by the corepressor complex Ssn6–Tup1 (Friesen et al. 1997).

2.2

Transcriptional Regulation During Meiosis in *S. pombe*

Microarray analyses in *S. pombe* have allowed the identification of several groups of genes whose transcription is coordinately induced during the processes of meiosis and sporulation. Mata et al. (2002) have reported that more than 50% of the genes are regulated in meiosis: almost 2000 genes are up-regulated at least twofold and more than 700 are induced more than fivefold (Mata et al. 2002). The successive transcriptional waves are under the control of transcription factors, induced at different times along the meiotic process, that establish four temporal classes of genes. The first group includes

genes involved in the nitrogen starvation and pheromone responses, such as *mat1⁺*, *mei2⁺*, *pat1⁺*, and *ste11⁺*. The second group (early genes) includes genes involved in premeiotic S-phase (e.g., *cig2⁺* and *cdc18⁺*), recombination (e.g., *rec12⁺*, *meu13⁺*, and *dmc1⁺*), and cohesion (e.g., *rec8⁺*). The group of middle genes is induced during the meiotic divisions and encodes cell cycle regulators, such as Cdc25 and Cdc13, condensins, kinesins, sporulation genes (e.g., *spo6⁺*), anaphase-promoting complex/cyclosome (APC/C) activators (e.g., *mfr1⁺* and *slp1⁺*), and APC/C regulators (e.g., *mes1⁺*). Late genes are induced after meiotic divisions, and are mainly involved in spore formation, maintenance of the dormant state of the spore, and germination.

Specific transcription factors control the expression of each wave of genes. For instance, many early genes are under the control of the transcriptional complex MBF (*MluI* binding factor), composed during meiosis of Cdc10, Res2, and Rep1 (Cunliffe et al. 2004; Ding and Smith 1998; Sugiyama et al. 1994; Zhu et al. 1997). Genes encoding these factors are also induced early on in meiosis. On the other hand, more than 50% of the middle genes contain FLEX sequences in their promoters, indicating that they are under the control of the meiotic forkhead transcription factor Mei4, which also strongly promotes its own transcription (Abe and Shimoda 2000). Regarding late genes, the Atf family of transcription factors, known to be involved in stress responses, regulates the expression of many of them. Approximately 55% of these late genes could be under the control of Atf21 and Atf31, explaining why spore formation is defective in the absence of these transcription factors (Mata et al. 2002).

Comparison of budding and fission yeast microarrays has revealed a group of commonly induced genes (< 100), including cell cycle regulators and genes involved in recombination and chromosome cohesion (Mata et al. 2002; Primig et al. 2000). In addition, it has been observed that organism-specific genes are better represented among genes induced at early stages of the meiotic process, perhaps to prevent meiosis between closely related species (Mata and Bahler 2003).

2.3

Expression Profiles During Mammalian Gametogenesis

In mammals, biochemical studies on gene expression along the process of gametogenesis have been hampered by the difficulties involved in obtaining populations enriched in germ cells (reviewed by Schlecht and Primig 2003, and Wrobel and Primig 2005). Schlecht et al. (2004), using highly enriched germ cell populations and somatic controls, identified 1268 loci differentially expressed in germ cells versus testicular somatic cells. More than 290 of them are as yet uncharacterized genes that could be involved in spermatogenesis and fertility (Schlecht et al. 2004). Recently, Pang et al. (2006) used purified germ cells to study stage-specific gene expression patterns during

spermatogenesis, and identified 160 genes differentially expressed in meiotic (pachytene spermatocytes) and postmeiotic (round spermatids) cells (Pang et al. 2006). The different pattern of transcription between germ and somatic cells is achieved by changes in both the transcription factors used and in the organization of chromatin, which makes the promoters of meiotic genes accessible (reviewed by DeJong 2006).

3

Cyclins and CDKs in Meiosis

As in mitosis, CDK–cyclin kinase activity regulates meiotic progression. In fact, the maturation or M-phase promoting factor (MPF) was first purified by its ability to induce meiotic maturation in *Xenopus* oocytes (Lohka et al. 1988). MPF is a heterodimer composed of the catalytic Cdc2 subunit and the regulatory cyclin subunit. The activation of Cdc2 requires the phosphorylation of Thr-161 by CAKs (CDK-activating kinases) and the dephosphorylation of Tyr-15 and Thr-14 by Cdc25 phosphatases. This inhibitory phosphorylation is carried out by the Wee1 and Myt1 kinases (reviewed by Moser and Russell 2000). In the meiotic cell cycle, the activity of this complex must be tightly regulated in order to induce an S-phase followed by two consecutive rounds of chromosome segregation, with no DNA synthesis between them. In higher eukaryotes, CDK activity in oocytes requires additional controls in order to ensure an initial arrest at prophase of meiosis I, before hormone stimulation, and a second arrest at metaphase I (many invertebrates) or metaphase II (vertebrates), which persists until fertilization.

3.1

CDK–Cyclin Regulation in Yeast Meiosis

3.1.1

CDK and Cyclins in *S. cerevisiae*

Initial experiments using thermosensitive mutants indicated that the budding yeast CDK, Cdc28, is essential for meiotic divisions but not for meiotic DNA replication (Shuster and Byers 1989). More recent studies using conditional *cdc28* mutants sensitive to chemical inhibitors have shown that Cdc28 is also necessary for premeiotic S-phase (Benjamin et al. 2003). Additionally, Ime1 induces the transcription of *IME2*, encoding a protein kinase that shows homology to Cdc28. Ime2 phosphorylates the Cdc28 inhibitor Sic1, triggering its degradation (Dirick et al. 1998). The mechanism by which Ime2 promotes Sic1 destruction is not known. In fact, a recent report by Sedgwick et al. (2006) suggests that this phosphorylation would be necessary, but not sufficient, to trigger Sic1 proteolysis (Sedgwick et al. 2006). Also, Ime2 inhibits the

APC/C complex, leading to the stabilization of B-type cyclins, which are required for S-phase and chromosome segregations (Bolte et al. 2002; reviewed by Honigberg 2004) (Fig. 2A).

The regulation of cyclins in *S. cerevisiae* is different in the meiotic cell cycle compared to the mitotic cell cycle. Of the six B-type cyclins functioning in mitosis (Clb1-6), only five play a role in meiosis (Clb1 and Clb3-6). Clb1, Clb3, and Clb4 are the most important cyclins for meiosis I entry and for the meiosis I to meiosis II transition. It is interesting to note that Clb2, the most important B-type cyclin for mitosis, seems to be unnecessary during meiosis. In contrast, *CLB1* is essential for meiosis and its deletion induces strong sporulation defects. *CLB3* and *CLB4* play a minor role, and the deletion of each of them enhances the sporulation defect of the *CLB1* deletion. These three cyclins, Clb1, Clb3, and Clb4, are induced before meiosis I, and are degraded after meiosis II. In contrast to the mitotic cycle, Clb3 and Clb4 are not synthesized in S-phase, suggesting that they are not involved in premeiotic DNA replication (Dahmann and Futcher 1995; Grandin and Reed 1993). In meiosis, this function is wholly carried out by Clb5 and Clb6. While *CLB5* *CLB6* deletion only induces a delay in mitotic S-phase initiation (Schwob and Nasmyth 1993), probably because the other Clb cyclins compensate, the same double mutant is unable to perform premeiotic DNA replication. Surprisingly, these cells proceed to meiotic divisions despite the absence of replication, indicating that the S/M checkpoint is not activated in this double mutant (Stuart and Wittenberg 1998).

In both budding and fission yeasts, CDK activity during meiotic prophase is low. In fact, this activity is the ultimate target of the meiotic recombination checkpoint in yeast, which prevents entry into meiosis I until completion of recombination and synapsis (see Sect. 5.1). However, recent experiments in budding yeast have suggested that this low Cdc28 kinase activity is required at early stages of meiotic recombination, regulating the formation of double-strand breaks (DSBs) (Henderson et al. 2006). In the restricted window when these events occur, Cdc28–Clb5 directly phosphorylates Mer2/Rec107, a protein required for DSB formation. This phosphorylation is critical for DSB formation, and it regulates the interaction of Mer2 with other factors required for the process (Henderson et al. 2006). Although a function for Clb5 in promoting DSB formation in meiosis had been established earlier (Smith et al. 2001), these experiments demonstrate that Cdc28–Clb5 activity is not limited to indirectly promoting DSB formation through the control of DNA replication (Cromie and Smith, this SERIES), but it plays a more direct role. Supporting a function for CDK activity in meiotic recombination in budding yeast, a role for Cdc28 in mitotic recombination, in the efficient resection of DSB ends, has been described (Aylon et al. 2004; Ira et al. 2004). Also, the meiosis-specific Rem1 cyclin is required for meiotic recombination in fission yeast (Malapeira et al. 2005), and the ribonucleotide reductase enzyme, required for DNA synthesis and repair, has been shown to be a substrate of

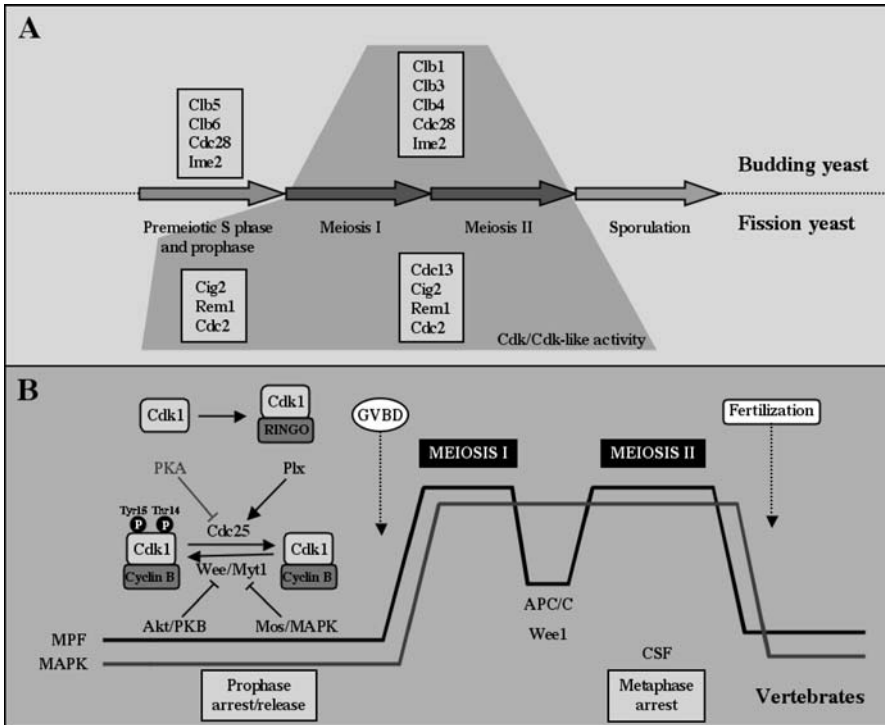


Fig. 2 Regulation of meiotic transitions in yeast and vertebrates. **A** Budding and fission yeasts. CDK activity fluctuates during meiosis in both budding and fission yeasts: it is low during premeiotic S-phase and prophase and reaches a peak during meiotic divisions. The kinases and cyclins that regulate these transitions in each yeast are represented. The CDKs (Cdc2 in fission yeast and Cdc28 in budding yeast) associate with different sets of cyclins during premeiotic S-phase and meiotic divisions. In budding yeast, Ime2, a CDK-like kinase that does not associate with cyclins, is required for both premeiotic S-phase and meiotic divisions, and it performs some of the mitotic Cdc28 functions during meiosis. See text for details. **B** Vertebrate oocytes. In vertebrate oocytes, meiosis progression is controlled by CDK–cyclin (MPF) and mitogen-activated protein kinase (MAPK) activities. The establishment of prophase arrest in vertebrate oocytes requires low MPF activity. When oocyte maturation occurs, several molecular pathways converge to activate CDK–cyclin complexes. In *Xenopus*, the negative regulation of Cdc25 by PKA is suppressed during meiotic resumption. The Polo kinase (Plx1) also contributes to Cdc25 activation. In addition, the MAPK/MEK/Mos/p90Rsk pathway is activated, leading to Myt1 inhibition. RINGO/Speedy also contributes to CDK activation in oocyte resumption. In starfish and mammalian oocytes, Akt/PKB phosphorylates Myt1 and inhibits its activity. While MAPK activity is high during meiotic divisions, MPF activity drops transiently between meiosis I and meiosis II. This decline is tightly controlled by APC/C and Wee1 in order to prevent a complete fall of CDK activity that could induce a round of DNA replication between meiotic divisions (see Sects. 3.4 and 4.2.2). In meiosis II, vertebrate oocytes arrest in metaphase due to the activity of cytotstatic factor (CSF), which keeps CDK activity high through APC/C inhibition (see Sect. 4.3). Fertilization induces the release of the oocyte from the metaphase arrest and the completion of meiosis

Cdc2/CDK2, pointing to this enzyme as a possible target for CDK activity during meiotic recombination (Chan et al. 1993, 1999; Holmberg et al. 2005).

3.1.2

CDK and Cyclins in *S. pombe*

In *S. pombe*, initial analyses using thermosensitive mutants revealed that Cdc2 is essential for meiosis II, since *cdc2* mutants at the restrictive temperature generated two-spore asci (Grallert and Sipiczki 1991; Nakaseko et al. 1984). However, a higher restrictive temperature induces an earlier block, before premeiotic S-phase, suggesting a role for Cdc2 kinase before DNA replication (Iino et al. 1995). In addition, the Cdc2-activating phosphatase Cdc25 is necessary for both meiosis I and meiosis II (Iino et al. 1995). On the other hand, the protein levels of the Wee1 kinase, a Cdc2 inhibitor, increase after premeiotic S-phase, and the Cdc2 Tyr-15 residue appears phosphorylated between premeiotic S-phase and meiosis I (Daya-Makin et al. 1992). Synchronous meiosis analyses have shown that this inhibitory phosphorylation disappears just before the first division, correlating with a strong increase in the Cdc2–Cdc13 kinase activity (Murakami and Nurse 1999). In addition, Cdc25 phosphatase, which catalyzes Cdc2 Tyr-15 dephosphorylation, increases after premeiotic S-phase (Iino et al. 1995). These results suggest that the Cdc2 kinase activity is also required in meiosis I, but a higher activity is necessary for the second division (Fig. 2A).

In *S. pombe*, M-phase cyclin Cdc13 begins to accumulate during premeiotic S-phase and remains high from the onset of meiosis I until the exit from meiosis II (Borgne et al. 2002). Experiments using a *cdc13* thermosensitive mutant indicate that *cdc13*⁺ is essential for both meiotic divisions, but not for premeiotic S-phase (Iino et al. 1995). In addition to Cdc13, other cyclins play a role in fission yeast meiosis. In the mitotic cell cycle, Cig1 and Cig2 control S-phase (Connolly and Beach 1994; Fisher and Nurse 1996; Martin-Castellanos et al. 1996; Obara-Ishihara and Okayama 1994). In meiosis, Cig2, but not Cig1, plays a dual role in premeiotic S-phase and in meiotic divisions (Borgne et al. 2002). Indeed, during meiosis, Cig2 is expressed in a biphasic manner. The first wave of expression occurs at the onset of premeiotic S-phase and the second one during the meiotic divisions. This second wave of expression is under the control of the meiotic transcription factor Mei4 (Borgne et al. 2002). The two waves of Cig2 induction correlate with peaks in Cig2-associated kinase activity. The first peak (S-phase) is lower than the second one (meiotic divisions), suggesting that in meiosis, as in the mitotic cell cycle, S-phase requires lower levels of CDK–cyclin kinase activity than M-phases (Borgne et al. 2002; Stern and Nurse 1996). In the absence of Cig2, entry into S-phase and meiosis I is delayed by approximately half an hour, but meiotic divisions do finally occur, probably because Cdc13 or other cyclins (e.g., Rem1) carry out the functions of Cig2.

Recently, a fission yeast meiosis-specific cyclin, Rem1, has been described (Malapeira et al. 2005). In the absence of Rem1, entry into meiosis I is delayed and meiotic recombination is impaired. Rem1 function in premeiotic S-phase becomes essential in the absence of Cig2, and the double mutant arrests meiotic progression before premeiotic DNA replication (Malapeira et al. 2005).

3.2

CDK–Cyclin Regulation in Higher Eukaryotes in Early Meiosis

Most results on the role of CDKs in mammalian gametogenesis come from studies using knockout mice (reviewed by Kierszenbaum 2006). Unexpectedly, the absence of a specific CDK or cyclin more often affects germ cells than somatic cells. It has been suggested that this lack of plasticity observed in reproductive cells would respond to the need to prevent the transmission of meiotic defects to the progeny (Pagano and Jackson 2004). In particular, the pool of CDK2 activity appears to be crucial for the meiotic processes that take place during prophase (i.e., recombination and synapsis).

Cdk2^{-/-} mice are viable, indicating that this cyclin-dependent kinase is not essential for cell proliferation, but they are infertile, because germ cells are unable to complete prophase (Berthet et al. 2003; Ortega et al. 2003). Both male and female knockouts show defects in synaptonemal complex (SC) organization and in the distribution of SYCP3, a protein involved in SC formation and synapsis (Ortega et al. 2003). However, CDK2 must have other targets because *Sycp3*^{-/-} females are partially fertile (Yuan et al. 2000). Female germ cells lacking *CDK2* progress to the diacytate stage, after prophase, and then undergo apoptosis. Spermatocytes undergo apoptosis earlier, at the pachytene stage of prophase, probably due to the activation of the pachytene checkpoint (Ortega et al. 2003; see Sect. 5.1). According to its meiotic role in prophase, CDK2 has been shown to colocalize with MLH1, a protein involved in reciprocal recombination, in mid-late pachytene (Ashley et al. 2001). It has also been observed along the asynapsed axes of X and Y chromosomes during the pachytene stage, a pattern described for other cell cycle regulators such as ATM, ATR, CHK1, and TopBP1 (Ashley et al. 2001; Flaggs et al. 1997; Keegan et al. 1996; Perera et al. 2004).

An essential function in mammalian meiosis has been described for cyclin A1 and cyclin E2. Both male and female mice lacking cyclin A1 (*Ccna1*^{-/-}) are healthy, and female fertility is not affected, but curiously male knockout mice are sterile (Liu et al. 1998; Salazar et al. 2003). *Ccna1*^{-/-} spermatocytes arrest before meiosis I with low MPF activity, and then undergo apoptosis. This phenotype resembles that of *Cdk2*^{-/-} male mice, but in this case axial element formation of pachytene chromosomes is not defective, although an incomplete desynapsis is observed in mid-diplotene nuclei (Liu et al. 1998). Human cyclin A1 is expressed at similar stages of spermatogen-

esis to *CDK2* (Wolgemuth et al. 2004). Study of knockout mice lacking E-type cyclins has revealed that neither cyclin E1 nor cyclin E2 is essential for normal development. However, again they show sex-specific differences: while females are fully fertile, male mice lacking cyclin E2 have reduced fertility (50% of them are sterile) and display testicular hypotrophy and very reduced sperm counts (Geng et al. 2003; Parisi et al. 2003).

In mouse, cyclin B3 is specifically expressed in leptotene and zygotene phases during meiotic prophase (Nguyen et al. 2002). Although the function of cyclin B3 in meiosis is not known, its pattern of expression suggests that it may regulate events occurring in meiotic prophase, such as recombination and synapsis. In addition, recent work has shown that downregulation of cyclin B3 at the zygotene–pachytene transition is required for normal spermatogenesis, since prolonging the expression of cyclin B3 until the end of meiosis leads to severe defects in spermatogenesis and a reduction in sperm counts (Refik-Rogers et al. 2006).

In *Drosophila*, a meiosis-specific Cdc25-type phosphatase, *Twine*, regulates CDK activity during gametogenesis. Expression of *twine* restricts to the male and female gonads, and the analysis of *twine* mutants indicates that it is required for both male and female fertility. In males, *twine* is required for meiotic entry, as the *twine*^{HB5} mutant arrest before meiotic divisions, with 4N nuclei. Nevertheless, as described for conditional *cdc2* alleles, these primary spermatocytes differentiate, and abnormal tetraploid spermatids are generated. In contrast to the situation in males, female oocytes enter meiosis, but they fail to arrest at metaphase of the first meiotic division, and this results in severe defects in oogenesis (Alphey et al. 1992; Courtot et al. 1992).

3.3

CDK Activity During Oocyte Maturation

In vertebrate oocytes, the transition from prophase arrest to metaphase arrest in meiosis II is known as oocyte maturation. During prophase arrest, CDK–cyclin activity is low due to the inhibitory phosphorylation of Cdk1 on Thr-14 and Tyr-15, carried out by the Myt1 or Wee1 kinases. The release from this cell cycle arrest occurs in response to hormonal stimulation, and requires the activation of several different molecular pathways (Fig. 2B).

In *Xenopus*, meiosis resumption requires new protein synthesis, specifically of cyclin B and c-Mos. Ablation of both inhibits oocyte maturation by progesterone stimulation, although blocking the synthesis of one of them does not (reviewed by Haccard and Jessus 2006). Mos induces activation of the MAPK/MEK/p90Rsk pathway, which leads to the phosphorylation and inhibition of Myt1 (Palmer et al. 1998). Another newly synthesized factor is RINGO/Speedy, a protein unrelated to cyclins that has been shown to bind and activate both Cdk1 and Cdk2 (Ferby et al. 1999; reviewed by Nebreda 2006). RINGO/Speedy is a potent inducer of meiotic maturation in the absence

of progesterone, and is required for timely progesterone-induced meiotic resumption (Ferby et al. 1999). Gutierrez et al. (2006) have recently found that RINGO expression has to be tightly regulated to ensure the maintenance of prophase arrest (Gutierrez et al. 2006). Multiple RINGO/Speedy homologs have been identified in mammalian cells. Some of them are highly expressed in testis and/or oocytes, suggesting that they may function during meiosis, as XRINGO (Cheng et al. 2005). Apart from the positive action of the above factors in oocyte resumption, PKA must be inactivated. In *Xenopus* oocytes arrested at prophase, PKA negatively regulates Cdc25C by phosphorylation on Ser-287 (the equivalent of Ser-216 in human Cdc25C) (Duckworth et al. 2002). This mechanism could be conserved since a recent work has demonstrated that Cdc25B and Wee1B are also substrates of PKA in mammalian oocytes (Han and Conti 2006). The activation of Cdc25C in *Xenopus* also requires the polo-kinase Plx1 (Qian et al. 2001). In starfish oocytes, protein kinase B (PKB) phosphorylates and downregulates Myt1, switching the balance between Cdc25 and Myt1 (Okumura et al. 2002), a pathway that could also be active in mammalian oocytes (Kalous et al. 2006). All these signaling pathways converge in the activation of Cdk1, where Cdc25 phosphatase counteracts the Wee1/Myt1 inhibition of CDK activity (reviewed by Kishimoto 2003).

Knockout mice lacking Cdc25B and Cdc25C have shown that these proteins are not essential for the cell cycle, perhaps because Cdc25A or other phosphatases can compensate their absence (Ferguson et al. 2005). However, Cdc25B has an essential function in the resumption of meiosis in oocytes arrested in prophase. In the absence of this Cdc25B, MPF activity remains low and germinal vesicle breakdown (GVBD, a hallmark of entry into meiosis) does not occur, leading to permanent prophase arrest and sterility in female knockout mice. This situation can be reversed by microinjection of Cdc25B mRNA into *Cdc25b*^{-/-}, but not when a mutant version of Cdc25B mRNA is used (Lincoln et al. 2002). These results indicate that Cdc25A and C, present in the oocytes, are unable to compensate for this meiotic function of Cdc25B.

3.4

Meiosis I to Meiosis II Transition

Meiosis is characterized by the absence of DNA replication between the two successive rounds of chromosome segregation, meiosis I and meiosis II. This is achieved by different strategies that are mainly aimed at preventing the complete fall of CDK–cyclin kinase activity between meiosis I and meiosis II.

3.4.1

Meiosis I to Meiosis II Transition in *Xenopus*

In *Xenopus* oocytes, the suppression of S-phase between meiosis I and meiosis II has been extensively studied. In this organism, Cdk1 activity drops tran-

siently after meiosis I and rises again at the onset of meiosis II. Furuno et al. (1994) have shown that the rapid reactivation of Cdk1 suppresses S-phase after meiosis I and prevents entry into interphase, and that the Mos/MAPK pathway is important for this reactivation of Cdk1, preventing the complete degradation of cyclin B (Furuno et al. 1994; Gross et al. 2000; see Sect. 4.2.2).

The absence of a general cell cycle regulator required for the interphase arrest could be involved in S-phase omission. It has been shown that the tight regulation of Wee1, at the level of both protein synthesis and activity, is essential for the orderly meiosis I to meiosis II transition. In *Xenopus*, it has been observed that the Cdk1-inhibiting kinase Wee1 is absent during oocyte maturation, and that it is specifically downregulated, primarily at the translational level, during oogenesis (Nakajo et al. 2000). The absence of Wee1 in meiosis I is a common feature of meiosis in many organisms, suggesting that this mechanism could be conserved across species (Daya-Makin et al. 1992; Kishimoto 1998; Mitra and Schultz 1996). These results are controversial, since other authors have shown that in *Xenopus* meiosis I extracts Wee1 is not absent but is insufficient to inactivate all residual Cdk1 activity³ (Iwabuchi et al. 2000).

In addition, the chromokinesin Xkid, required to align chromosomes on the metaphase plate, is necessary for Cdk1/cyclin B reactivation after meiosis I, maybe by promoting cyclin B synthesis. Supporting a role for this factor in the meiosis I to meiosis II transition, it has been shown that Xkid-depleted cells enter an interphase state and undergo DNA replication (Perez et al. 2002).

3.4.2

Meiosis I to Meiosis II Transition in Other Eukaryotes

In budding yeast, exit from mitosis requires the cooperation of two pathways that control the release and activation of the phosphatase Cdc14: the FEAR (CDC fourteen early anaphase release) and the MEN (mitotic exit network; reviewed by Simanis 2003). One of the mechanisms that could regulate the transition from meiosis I to meiosis II is use of the FEAR to inactivate CDK after meiosis I. The FEAR network is dispensable in mitosis but is essential for exit from meiosis I (Kamieniecki et al. 2005). It has been suggested that FEAR induces a partial reduction in CDK activity, enough to disassemble the meiosis I spindle and prevent entry into G₁ phase, whereas for exit from mitosis a more complete CDK inactivation is necessary that can only be carried out by the MEN (Kamieniecki et al. 2005). Recently, a mechanism for the role of separase in FEAR and mitosis exit has been established (Queralt et al. 2006). Moreover, the requirement of separase for the exit from meiosis I

³ As CDKs and Wee1 can inactivate one another by mutual phosphorylation, the dominating residual activity depends on the actual protein ratio.

has also been observed in mouse oocytes, where a catalytically inactive version of separase is able to block polar body extrusion (Kudo et al. 2006). This new function of separase seems to be associated with its ability to bind Cdk1, since abrogation of this physical interaction also causes a defect in polar body extrusion (Gorr et al. 2006; reviewed by Terret and Jallepalli 2006).

4

Control of APC/C Activity in Meiosis

Since the discovery of the E3 ubiquitin ligase APC/C (anaphase-promoting complex/cyclosome) 10 years ago, much work has been done in the field of proteolysis-regulated cell cycle events. The temporal and spatial regulation of protein degradation is tightly controlled during the cell cycle, not only in mitotic cells but also in meiosis. In meiosis, there are specific APC/C activators and regulators that allow the normal cell cycle to accommodate the new meiotic requirements. Furthermore, the proteasome (the proteolytic complex that degrades ubiquitinated proteins) undergoes dramatic changes in its localization during meiosis (Wilkinson et al. 1998).

4.1

APC/C Activity Must be Tightly Controlled in Meiotic Prophase

Meiotic prophase (the time between the completion of DNA synthesis and the first meiotic division) is expanded, as compared to mitotic cells. This is a key characteristic of meiosis, since during this period of time the nuclear architecture changes in order to help homologous chromosomes to align and pair. It is also during prophase when the physical exchange between homologous chromosomes occurs, ensuring the genetic diversity generated in meiosis. In vertebrates, prophase may take years, since oocytes spend long periods of time in the ovaries before hormones induce their maturation. How is the meiotic prophase established? At least two mechanisms involving APC/C regulation have been described recently (Irniger 2006; Oelschlaegel et al. 2005; Penkner et al. 2005; Reis et al. 2006).

Securin is a known mitotic APC/C substrate that maintains sister chromatids together by inhibition of separase (the protease that actually cleaves cohesin) until the metaphase–anaphase transition, when APC/C targets it for proteasome destruction (Ciosk et al. 1998; Cohen-Fix et al. 1996; Funabiki et al. 1996a,b; Uhlmann et al. 2000; Waizenegger et al. 2000). Securin is also present in meiotic nuclei and is successively diminished at the onset of each meiotic division (Salah and Nasmyth 2000). Stepwise cleavage of the meiosis-specific cohesin subunit Rec8 triggers loss of cohesion, and therefore chromosome segregation during meiosis I and meiosis II (Buonomo et al. 2000; Kitajima et al. 2003; reviewed by Petronczki et al. 2003; Tanaka and

Watanabe, this BOOK). Thus, inhibition of securin degradation prevents Rec8 cleavage and preserves cohesion.

In budding yeast, Aml1 (a Cdc20 family member) is a meiosis-specific APC/C activator that was first described as a protein involved in the degradation of Clb1 (Cooper et al. 2000). Although Aml1 is required for the first meiotic division, it is expressed earlier in the meiotic program and it continuously binds to APC/C (Cooper et al. 2000; Oelschlaegel et al. 2005). However, it is inactive due to inhibition by Mnd2, a subunit of APC/C (Oelschlaegel et al. 2005; Penkner et al. 2005). This inhibition is crucial for the prevention of premature sister chromatid separation during S-phase and prophase, since in *mnd2* mutants Rec8 is rapidly cleaved during this period due to the increased turnover of securin (Fig. 3A: prophase).

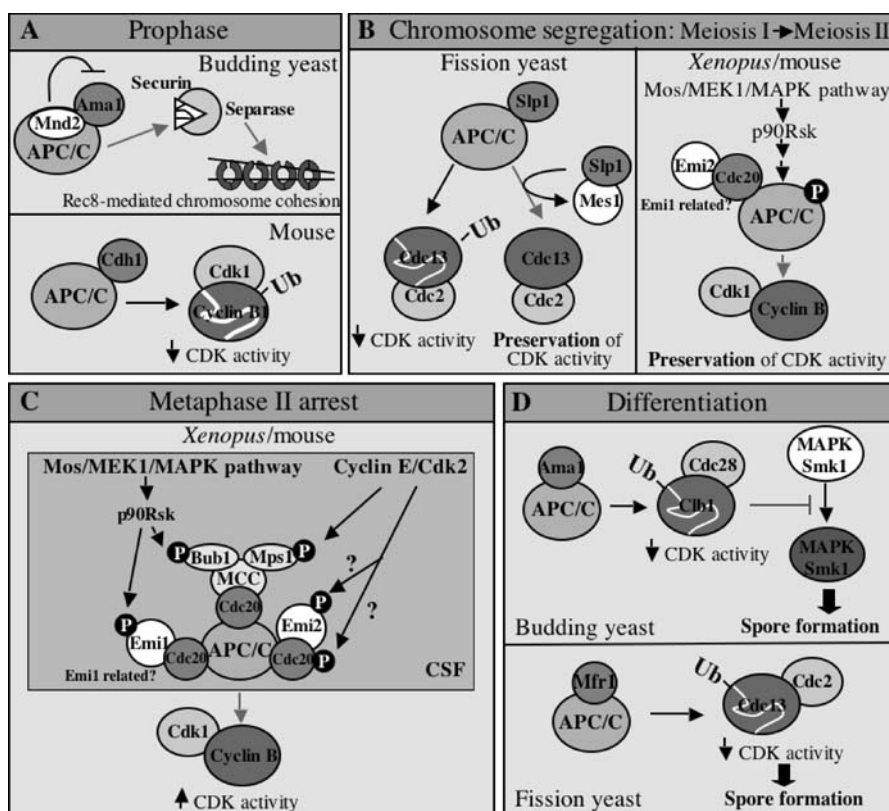
The deletion of *AMA1* and not of other APC/C activators present in meiosis (*CDC20* and *CDH1*) rescues the *mnd2* phenotype and restores securin levels, and Mnd2 inhibits exclusively the APC/C–Aml1 holoenzyme in vitro (Oelschlaegel et al. 2005; Penkner et al. 2005). These results explain the similar phenotype of *mnd2* and *rec8* mutants. *rec8*-defective cells are impaired in SC formation and DSB repair (Klein et al. 1999), the same phenotypes as those observed in *mnd2* mutants (Oelschlaegel et al. 2005; Penkner et al. 2005; Rabitsch et al. 2001).

Another APC/C activator, Cdh1, is required to maintain mouse oocytes in prophase (Reis et al. 2006). In contrast to Aml1 in budding yeast, Cdh1 is not meiosis-specific and it is required for cyclin B1 degradation at the end of mitosis and G₁ in different organisms (Harper et al. 2002; Peters 2002). A high frequency of GVBD is obtained by injecting oocytes in prophase with either a stabilized cyclin B1 version or *CDH1* morpholino oligonucleotides to knock-out *CDH1* function (Reis et al. 2006). Moreover, Cdh1 inhibition dramatically increases the otherwise moderate rate of GVBD achieved by microinjection of the wild-type cyclin B1 (Reis et al. 2006). Thus, Cdh1 must play a role in keeping cyclin B1 levels low during prophase of mammalian oocytes (Fig. 3A: prophase). A recent report has also established a role for Cdh1 in maintaining cyclins at low levels in postmitotic neurons to prevent aberrant entry into S-phase and apoptosis (Almeida et al. 2005), suggesting a conserved function of Cdh1 in the control of cyclin levels in situations in which cell cycle progression must be restrained. In addition to APC/C regulation, the regulation of Cdc25/Wee1 is also involved in prophase establishment (see Sect. 3.3).

4.2

APC/C Activity Must be Modulated During Chromosome Segregation

As in the mitotic cycle, chromosome segregation during meiosis requires the loss of sister chromatid cohesion, which is achieved by controlled degradation of the meiosis-specific cohesin subunit Rec8 (Buonomo et al. 2000; Kitajima et al. 2003; Kudo et al. 2006; Lee et al. 2006; reviewed by Petronczki et al.



2003). In contrast to mitotic cells, cohesin degradation occurs twice during meiosis. First in meiosis I, when cohesion is lost in the chromosome arms to allow chiasmata resolution and homologous chromosome segregation, and then in meiosis II, when residual cohesion at sister centromeres is lost to allow the quasi sister chromatids⁴ to segregate to opposite poles of the cell (see Tanaka and Watanabe, this volume). As in mitotic cells, degradation of cohesins in meiosis in most organisms requires APC/C activity and securin destruction/separase activation (Davis et al. 2002; Herbert et al. 2003; Kudo et al. 2006; Lee et al. 2006; Siomos et al. 2001; Terret et al. 2003). *Xenopus* oocytes may be an exception since APC/C inactivation or securin stabilization does not block meiosis I progression (Peter et al. 2001; Taieb et al. 2001); however, securin disappears both in anaphase I and in anaphase II during oocyte maturation and activation (Fan et al. 2006). The APC/C activator Cdc20 is required for the degradation of securin in mitotic and probably in meiotic cells (Lim et al. 1998; Salah and Nasmyth 2000; Schott and Hoyt 1998; Visintin et al. 1997).

⁴ At this stage, the meiotic chromatids are only sisters close to the centromeres, but are scrambled by crossovers further out.

◀ **Fig. 3** APC/C regulation in meiosis. **A** Prophase. The inhibition of degradation of cohesins, and destruction of cyclin B1 are important for prophase establishment. In budding yeast, Mnd2 inhibits the activation of APC/C by Ama1 to prevent securin destruction, and hence Rec8 degradation and release of cohesion. In mouse oocytes, Cdh1 activates APC/C and promotes cyclin B1 degradation. **B** Chromosome segregation. Preservation of CDK activity between the two chromosome segregations is an important feature of meiosis. In fission yeast, Mes1 may function as a competitive inhibitor of APC/C-Slp1, preventing the total degradation of Cdc13 and preserving CDK activity. In *Xenopus*, the Mos/MEK1/MAPK pathway is required to inhibit replication between meiosis I and meiosis II. The pathway is involved in the inhibition of cyclin B degradation, and its activation correlates with APC/C phosphorylation. In addition, Emi2, the inhibitor of the APC/C-Cdc20 holoenzyme, is also required for proper meiosis I to meiosis II transition in *Xenopus* and mouse. Emi1 or an Emi1-related protein could also be required. **C** Metaphase II arrest. The Mos/MEK1/MAPK/p90Rsk pathway and cyclin E/Cdk2 cooperate to establish CSF arrest through APC/C inhibition. p90Rsk binds and phosphorylates Emi1 in vitro, and this phosphorylation enhances the binding of Emi1 to the APC/C activator Cdc20, although the role of Emi1 in CSF needs to be addressed with specific reagents both in *Xenopus* and in mouse. The Bub1 kinase is also required for CSF arrest in *Xenopus* and is phosphorylated in vitro by the Mos/MEK1/MAPK/p90Rsk pathway. In the spindle assembly checkpoint (SAC), Bub1 is required to load the mitotic checkpoint complex (MCC), which binds to Cdc20 and prevents Cdc20 from activating APC/C. The action of cyclin E/Cdk2 over APC/C-Cdc20 is also indirect and SAC-mediated by phosphorylation of Mps1. Emi2 is a conserved bona fide APC/C-Cdc20 inhibitor involved in CSF arrest in *Xenopus* and mouse that seems to work independently of the Mos/MEK1/MAPK/p90Rsk pathway; the connection with cyclin E/Cdk2 remains to be established. A postulated cyclin E/Cdk2-dependent phosphorylation, presumably of Cdc20 or another as yet unknown APC/C activator, could also contribute to metaphase II arrest in *Xenopus*. **D** Differentiation. In budding yeast, the meiosis-specific APC/C activator Ama1 downregulates CDK activity and allows activation of the MAPK Smk1, a meiosis-specific kinase required for spore formation. In fission yeast, low CDK levels are also required for differentiation and this is achieved by Mfr1-mediated Cdc13 degradation. *Black lines* indicate participation in the process. *Gray lines* indicate inhibition of such participation

The spindle assembly checkpoint (SAC) is a surveillance mechanism that senses defects in the attachments of kinetochores to microtubules (reviewed by Nasmyth 2005, and Taylor et al. 2004). In mitotically dividing yeast cells, this mechanism is essential only when the cell faces chromosome-microtubule anchoring problems and it directly inhibits the activation of APC/C-Cdc20. APC/C-Cdc20 inhibition is mediated by direct binding of a protein complex containing Mad2 to Cdc20. This inhibition blocks the onset of anaphase (securin destruction) to avoid chromosome missegregation. However, during meiosis the SAC is important for accurate chromosome segregation in an unperturbed meiosis (Bernard et al. 2001; Shonn et al. 2000), showing that metaphase delay is an intrinsic feature of the meiotic program. This could reflect the complexity involved in correctly orienting homologous chromosomes in metaphase of meiosis I as compared to mitosis (reviewed by Marston and Amon 2004, and Petronczki et al. 2003; see Tanaka and Watan-

abe, this volume). In meiosis, not only do sister kinetochores have to bind to microtubules from the same pole of the cell (co-orientation), but homologous kinetochores must bind to microtubules from opposite poles (bi-orientation). The meiosis-specific complex monopolin, and Mad2 (a SAC component) in budding yeast, and the meiosis-specific protein Moa1, and Bub1 (a SAC component) in fission yeast are required for this kinetochore behavior (Bernard et al. 2001; Katis et al. 2004; Lee et al. 2004; Rabitsch et al. 2003; Shonn et al. 2000; Toth et al. 2000; Yokobayashi and Watanabe 2005). The SAC also plays an important role in meiosis in higher eukaryotes. In mammalian meiosis a correlation between low levels of SAC components and oocyte aging and infertility has been reported (reviewed by Homer 2006), and in *Drosophila*, SAC components are required for a proper completion of meiosis (Fischer et al. 2004; Gilliland et al. 2005).

Meiosis and sex-specific APC activators have been described in *Drosophila* (Chu et al. 2001; Jacobs et al. 2002). In the case of female meiosis, Cortex, together with the ubiquitous APC activator Fzy (*Drosophila* Cdc20), is required for anaphase progression both in meiosis I and meiosis II, although the requirement for meiosis II seems to be greater (Swan and Schüpbach 2007). During meiosis II, Cortex cooperates in the destruction of cyclin B associated to the meiotic spindle; interestingly, the timing and location of this destruction differs from the cyclin B destruction promoted by Fzy, indicating that the two APC activators are required at different stages of anaphase progression (Swan and Schüpbach 2007).

In *Xenopus* oocytes, complete abrogation of cyclin B/Cdk1 activity at meiosis I blocks the segregation of sister chromatids and promotes a new round of DNA synthesis (Furuno et al. 1994; Iwabuchi et al. 2000; Nakajo et al. 2000). These works uncovered the preservation of CDK activity between the two successive rounds of chromosome segregation as an important feature of meiosis that is conserved in other eukaryotes (Blanco et al. 2001; Borgne et al. 2002; Dekel 2005). The preservation of cyclin B/Cdk1 activity is achieved by different mechanisms: inhibition of a complete cyclin B degradation, increased cyclin B synthesis, and the control of its associated kinase activity (Furuno et al. 1994; Gross et al. 2000; Hochegger et al. 2001; Izawa et al. 2005; Nakajo et al. 2000; Tung and Jackson 2005). In this section we shall focus on cyclin B degradation since APC/C regulation is involved in this mechanism, while the control of CDK activity has been discussed previously (see Sect. 3.4).

4.2.1

APC/C Regulation in Meiosis I to Meiosis II Transition in *S. pombe*

In fission yeast, a meiosis-specific APC/C inhibitor, Mes1, is present from the end of metaphase I to early anaphase II (Izawa et al. 2005; Mata et al. 2002; reviewed by Peters 2005, and Irniger 2006). Mes1 is essential for meiosis II and

mes1 mutants arrest prior to anaphase II with two separate nuclei (Shimoda et al. 1985). Recently, Izawa and coworkers (2005) have shown that while Cdc13 (fission yeast cyclin B1) is maintained until anaphase II in normal meiosis, in *mes1* mutant cells Cdc13 disappears at anaphase I. The *mes1* arrest can be bypassed by increasing the levels of B-type cyclins or with a loss-of-function version of Slp1 (fission yeast Cdc20), indicating that the main function of Mes1 is to preserve sufficient cyclin levels between the meiotic nuclear divisions. Furthermore, Mes1 and Slp1 counteract each other in different *in vivo* assays and bind to each other *in vitro*. Indeed, Mes1 inhibits the degradation of Cdc13 by APC/C–Slp1 in a dose-dependent manner in both yeast and *Xenopus* extracts.

The N-terminus region of Mes1 contains putative KEN and D-boxes and is required for binding to Slp1. These boxes are found in APC/C substrates and are required for their degradation (Harper et al. 2002; Irniger 2006). Indeed, in Cdc13 similar sequences are also required for binding to Slp1, which has led to the proposal that Mes1 could function as a competitive inhibitor of APC/C–Slp1. Mes1 could be an APC/C substrate that “distracts APC/C attention” from Cdc13 (Fig. 3b: chromosome segregation). Mes1 shares no obvious homology with other APC/C inhibitors known to bind to Cdc20 (Emi1 and Mad2), and these inhibitors bind to Cdc20 in a different manner. Mes1 interacts with the C-terminus region of Slp1/Cdc20, while Mad2 and Emi1 require an intact Cdc20’s N-terminus to do so (Hwang et al. 1998; Kim et al. 1998; Reimann et al. 2001). Indeed, crystal and NMR structures have shown that Mad2 binds Cdc20’s N-terminus (Luo et al. 2000; Sironi et al. 2002; reviewed by Nasmyth 2005). However, it is worth mentioning that Emi1 has been recently shown to function in mitotic cells as a pseudosubstrate inhibitor competing for D-box binding to APC/C (Miller et al. 2006).

4.2.2

APC/C Regulation in Meiosis I to Meiosis II Transition in *Xenopus*

In *Xenopus* it has been proposed that the preservation of low cyclin B levels at the end of meiosis I also requires the inhibition of cyclin B degradation (Gross et al. 2000). Oocytes entering S-phase after meiosis I by inhibition of the MAPK pathway with U0126 exhibit very low levels of cyclin B and a high mobility of an APC/C subunit that has been correlated with an active APC/C complex. Interestingly, in U0126-treated oocytes injected cyclin B is degraded more rapidly than in untreated oocytes. Activation of the MAPK signaling pathway, using a constitutively activated MAPK target p90Rsk, restores cyclin B accumulation as well as the low mobility of the APC/C subunit. It is still unknown whether APC/C is a substrate of p90Rsk (Fig. 3B: chromosome segregation).

A more direct link between APC/C inhibition and the meiosis I to meiosis II transition in *Xenopus* has been established recently (Tung and Jackson 2005).

Emi1 is an APC/C inhibitor required to keep APC/C inactive during the S and G₂ phases of the cell cycle (Reimann et al. 2001). In addition to this mitotic function, depletion of Emi1 in meiosis I oocytes induces a loss of cyclin B levels and entry into S-phase, indicating that it is also required to establish a proper meiosis I to meiosis II transition (Fig. 3b: chromosome segregation). The Emi1 depletion phenotypes are blocked by prior ablation of XFzy (*Xenopus* Cdc20), the inhibition of ubiquitination by addition of methyl ubiquitin, or the expression of a nondegradable version of cyclin B. The exclusive involvement of Emi1 in the process should be considered with caution because the antibodies used in that study recognize both Emi1 and a related APC/C inhibitor, Erp1/Emi2 (hereafter Emi2) (Schmidt et al. 2005; Tung et al. 2005; see below). Indeed, recent work shows that *Xenopus* Emi2 appears at the end of meiosis I, and when Emi2 expression is inhibited by morpholino oligos, meiosis II entry is prevented and DNA synthesis occurs (Liu et al. 2006; Ohe et al. 2007). Moreover, Emi2 is conserved in mammals (Shoji et al. 2006). Mouse Emi2 is also required to establish a proper meiosis I to meiosis II transition, since abrogation of Emi2 expression impairs metaphase II spindle assembly and decondenses chromatin, phenotypes that can be restored by Emi2 addition or expression of a nondegradable cyclin B1 version (Madgwick et al. 2006).

Local preservation of cyclin B at the spindle in meiosis I could also be a mechanism to preserve CDK activity between meiosis I and meiosis II. Localization of cyclin B in the spindle of anaphase I has been observed in *Drosophila* and, as mentioned above, the local destruction of this cyclin B in the spindle of meiosis II is required to drive anaphase II (Swan and Schüpbach 2007). In fission yeast, Cdc2 has also been observed to decorate the meiotic spindle in meiosis I, and Cdc2 has been proposed as a target of the meiotic spindle checkpoint (Decottignies et al. 2001; Yamaguchi et al. 2003).

4.3

APC/C Activity Must be Kept Low to Allow Vertebrate Oocytes to Arrest in Metaphase II

In vertebrate eggs, high levels of cyclin B/Cdk1 activity are required to maintain final metaphase II arrest after oocyte maturation and prior to fertilization/activation (reviewed by Tunquist and Maller 2003). To establish metaphase II arrest, different independent pathways, all of them contributing to what is known as cytostatic factor (CSF), could impinge on APC/C–Cdc20 inhibition and hence the preservation of cyclin B levels and its associated kinase activity (Fig. 3c: metaphase II arrest). The contribution of different pathways was observed in experiments with *Xenopus* oocyte cycling extracts in which the activation of the Mos/MEK1/MAPK/p90Rsk pathway caused metaphase arrest in the absence of cyclin E/Cdk2, and conversely, an active cyclin E/Cdk2 complex caused a metaphase arrest in the absence of Mos (Tunquist et al. 2002). Similarly, a metaphase arrest is observed by depletion

of Xfzy in cycling extracts, whereas the depletion of Xfzy in CSF extracts blocks the exit from metaphase II induced by activation (Lorca et al. 1998). Therefore, at least two pathways cooperate to establish CSF arrest: MAPK and cyclin E/Cdk2. What is the connection between these pathways and the inhibition of APC/C–Xfzy?

The APC/C inhibitor Emi1 could also contribute to CSF arrest in *Xenopus*, since immunodepletion of Emi1 from CSF extracts causes degradation of cyclin B and release from CSF arrest (Reimann and Jackson 2002) (Fig. 3c: metaphase II arrest). However, there have been some concerns about the specificity of the antibodies used, and the presence or absence of Emi1 in metaphase II-arrested oocytes (Ohsumi et al. 2004), making the involvement of Emi1 in CSF arrest a controversial issue. Nevertheless, mouse oocytes arrested in metaphase II do contain Emi1, and a role for Emi1 in CSF has been proposed, as well as a connection with the MAPK pathway (Paronetto et al. 2004). Emi1 binds to p90Rsk in extracts from tissue culture cells expressing both proteins. p90Rsk is able to phosphorylate Emi1 in vitro, and this phosphorylation enhances the binding of Emi1 to Cdc20. Moreover, microinjection of an N-terminal truncated Emi1 version that acts as a competitor for p90Rsk induces a defect in anaphase II spindle or a defect in CSF arrest. Similar results have been obtained by depleting Emi1 using RNAi. These in vivo experiments showed that Emi1 plays a role in meiosis II progression and CSF arrest. Nevertheless, reagent specificity is still a concern for this in vivo work since Emi2 is also conserved in mice; the Emi1 and Emi2 proteins are 39% identical in their C-terminal part (Schmidt et al. 2005), and the reagents used recognize this part of the gene or they are N-terminal truncated versions containing the conserved C-terminal domain intact.

Recent work has pointed to Emi2 as a bona fide APC/C–Cdc20 inhibitor involved in CSF arrest (Fig. 3c: metaphase II arrest). Emi2 appears during *Xenopus* oocyte maturation at the end of meiosis I, is stable in CSF extracts, and is abruptly degraded after fertilization via the SCF ubiquitin ligase (Liu et al. 2006; Ohe et al. 2007; Schmidt et al. 2005; Tung et al. 2005; Zachariae 2005). Immunodepletion of Emi2 releases the metaphase arrest of CSF extracts, which correlates with a drop in Cdk1 kinase activity and chromatin decondensation. The antibodies used in that work react specifically with Emi2 and not with the related protein Emi1, which makes the results obtained reliable. Adding Emi2 back to the extracts restores CSF arrest (Schmidt et al. 2005). Emi2 is a phosphoprotein that seems to function independently of the MAPK/p90Rsk pathway (Ohe et al. 2007; Schmidt et al. 2005). Whether Emi2 is a substrate for cyclin E/Cdk2 is still unknown. However, Emi2 is a substrate for Plx1 kinase, which triggers Emi2 for degradation, causing release from CSF arrest after fertilization (Rauh et al. 2005; Schmidt et al. 2005; Tung et al. 2005). This role of Emi2 in CSF arrest is also conserved in mammals, where Emi2 depletion/addition causes similar phenotypes to those described in *Xenopus* (Shoji et al. 2006).

A substrate of the MAPK/p90Rsk pathway is the kinase Bub1, a component of the SAC. Activation of the MAPK pathway in *Xenopus* cycling extracts establishes CSF arrest. This is prevented when Bub1 is immunodepleted, and readdition of wild-type Bub1, but not of a kinase-dead version, restores the ability of the MAPK pathway to establish CSF arrest (Tunquist et al. 2002). Immunodepletion of Mad1 or Mad2 also blocks the establishment of metaphase II arrest (Tunquist et al. 2003). Moreover, p90Rsk is able to phosphorylate and activate Bub1 in vitro (Schwab et al. 2001). Thus, the connection between the MAPK pathway and APC/C inhibition could be indirect and SAC-mediated (Fig. 3c: metaphase II arrest).

Since G₁-cyclin/Cdk complexes inhibit APC/C during the mitotic cycle by phosphorylation and dissociation of the APC/C activator Cdh1 (Blanco et al. 2000; Jaspersen et al. 1999; Zachariae et al. 1998), it is possible that the same situation could apply to the control of cyclin E/Cdk2 over APC/C in meiosis (Fig. 3c: metaphase II arrest). This mechanism should work through Cdc20, or a hitherto unknown meiosis-specific APC/C activator (as Aml1 and Mfr1), since at least *Xenopus* oocytes do not contain Cdh1 (Lorca et al. 1998). A mechanism for cyclin E/Cdk2 to establish CSF arrest has been recently uncovered (Grimison et al. 2006). The CSF arrest imposed by an active cyclin E/Cdk2 complex—but not by Mos—requires the SAC kinase Mps1, a cyclin E/Cdk2 substrate also involved in centrosome duplication (Abrieu et al. 2001; Fisk and Winey 2001; Grimison et al. 2006). As for the MAPK pathway, APC/C inhibition by cyclin E/Cdk2 could be indirect and SAC-mediated.

Tight control of APC/C activation is crucial during meiosis. Premature loss of sister chromatid cohesion at centromeres in prophase or in meiosis I could lead to aberrant segregations producing gametes with an abnormal number of chromosomes (aneuploidy). Most of these numerical chromosome defects will be deleterious, as shown by the fact that a high percentage of human miscarriages show this type of defect. In addition, defects in metaphase arrest at the end of egg maturation cause parthenogenetic development in unfertilized eggs.

4.4

APC/C Must be Kept Active in Order to Allow Differentiation

The meiotic program ends with the transcriptional induction of genes involved in the formation of specialized cell types: spores in yeasts and gametes in animals (Chu et al. 1998; Mata et al. 2002; Perezgasga et al. 2004; Primig et al. 2000; Schlecht et al. 2004; see Sect. 2). At least in lower eukaryotes, the coordination between the end of meiosis and the differentiation program seems to be orchestrated by APC/C regulation. In budding and fission yeasts, mutations in meiosis-specific APC activators disrupt this coordination (Asakawa et al. 2001; Blanco et al. 2001; Cooper et al. 2000).

Budding yeast mutants in the APC/C activator Aml1 do not form spores. Several genes transcribed late during the meiotic program, including some already known to be required for spore formation, are downregulated in the mutant. Although the prospore membrane forms properly, as assayed by electron microscopy, no mature spores are observed (Cooper et al. 2000). Recently, a more direct connection between Aml1 and spore formation has been established (McDonald et al. 2005). In *aml1* mutants the activation of Smk1, a meiosis-specific MAPK homolog that regulates spore formation, is impaired.

A similar phenotype to *aml1* mutants has been described for the fission yeast meiosis-specific APC/C activator Mfr1/Fzr1 (Asakawa et al. 2001; Blanco et al. 2001). *mfr1/fzr1* mutants progress through meiosis with normal kinetics and meiotic segregation of chromosomes is also normal. However, the spore envelope is defective and only few and aberrant spores are formed. The mitotic cyclin Cdc13 is stabilized in *mfr1/fzr1* mutants and the expression of a nondegradable Cdc13 version mimics the *mfr1/fzr1* phenotype (Blanco et al. 2001). This experiment indicates that high levels of the mitotic cyclin Cdc13 and CDK activity are not compatible with terminal spore differentiation. The spore formation defect of *aml1* mutants can be restored by lowering CDK activity, and CDK activity negatively regulates Smk1 activation (McDonald et al. 2005), suggesting that the defect could be explained—as in fission yeast—by an increased level of cyclins (Fig. 3d: differentiation). The control of Cdc13 levels at the end of meiosis in fission yeast is mainly exerted by the APC/C–Mfr1 holoenzyme, since *cdc20⁺* is not expressed in late meiosis (Mata et al. 2002) and deletion of the *S. pombe cdh1⁺* homolog (*ste9⁺/srw1⁺*) does not affect sporulation (Blanco et al. 2001).

5 Checkpoints in Meiosis

Checkpoints are surveillance mechanisms that ensure the correct completion and order of different cell cycle events (Hartwell and Weinert 1989). Meiotic cells face similar problems to mitotic cells, in addition to new ones arising from their particular nature. Accordingly, the checkpoints used during the mitotic division are also used during meiosis. For example, DNA damage or problems in replication may occur either in a mitotic or in a meiotic cell; the surveillance mechanisms that deal with the damage, stop cell cycle progression, and fix the lesion must be operative in both situations (Murakami and Nurse 1999; Stuart and Wittenberg 1998; reviewed by Nyberg et al. 2002). In addition, fission yeast meiotic cells have a backup DNA replication checkpoint that works at least when the main one is not operative (Murakami and Nurse 1999). Unlike the mitotic checkpoint and the main meiotic one, this alternative checkpoint does not control the activity of cyclin B/Cdk1 complexes and arrests cells in metaphase I instead of prophase (Murakami and Nurse 1999). In other cases,

meiosis-specific proteins replace their mitotic counterparts. This is the case of mammals, where a meiosis-specific member of the p53 family, p63, acts in a conserved process of monitoring the integrity of the female germ line, where p53 function is restricted to somatic cells (Suh et al. 2006).

Since defects in spindle formation or the attachment of kinetochores to the spindle are problems intrinsic to chromosome segregation, something that occurs both in mitotic and meiotic cells, the SAC is also operative in meiotic cells (see Sect. 4.2). The SAC works through a protein complex (Bub3/Mad2/Mad3) known as the mitotic checkpoint complex (MCC), which binds to unattached kinetochores. MCC also binds to Cdc20 and prevents Cdc20 from activating APC/C, thereby protecting the destruction of securin and the release of cohesion. Interestingly, at least in budding yeast and maybe in mammals, the meiotic function of Mad3 seems to be related to the delay in prophase progression rather than to chromosome segregation (Cheslock et al. 2005).

5.1

The Recombination Checkpoint

Meiotic cells also have to deal with new situations that need to be properly coordinated with meiotic progression, for example, the presence of DSBs required for recombination. In budding and fission yeasts, DSB formation is linked to the progression of premeiotic DNA replication. Situations that block DNA synthesis after origin firing, but preserve a normal DNA damage checkpoint response, also block DSB formation (Borde et al. 2000; Tonami et al. 2005). This checkpoint ensures that replication will not progress through unrepaired DNA (the coupling of premeiotic DNA replication and DSB formation is discussed by Cromie and Smith, this SERIES). In addition, cells need to properly synapse homologous chromosomes and recombine them before the first meiotic division occurs. This ensures the formation of physical links between homologous chromosomes (chiasmata), and therefore reductional chromosome segregation (meiosis I) will successfully take place due to the tension generated by the chiasmata in the bipolar spindle. A meiosis-specific surveillance mechanism that inhibits meiotic progression in response to defects in synapsis and recombination intermediates was first described in budding yeast. Mutants in a meiosis-specific Rad51 homolog (*DMC1*) or in a SC component (*ZIP1*) arrest in late prophase (Bishop et al. 1992; Sym et al. 1993). This arrest requires the checkpoint proteins Rad24, Rad17–Ddc1–Mec3 in a complex, and Mec1, all of them also involved in the mitotic DNA damage checkpoint (Hong and Roeder 2002; Lydall et al. 1996). Interestingly, the *dmc1* and *zip1* arrests are also overcome by deleting *SPO11*, the endonuclease required to initiate recombinogenic DNA DSBs (Bishop et al. 1992; Sym et al. 1993). Since recombination and synapsis are linked in budding yeast, these experiments suggested that the accumulation of recombination intermediates was indeed activating the checkpoint. This checkpoint was named

the pachytene checkpoint, since it is at this time during prophase when meiotic cells arrest in response to the defects (reviewed by Roeder and Bailis 2000). Over time, it has been found to be a widespread checkpoint, present from fission yeast to higher eukaryotes (Abdu et al. 2002; Jackson et al. 2006; MacQueen and Villeneuve 2001; Perez-Hidalgo et al. 2003; Roeder and Bailis 2000; Shimada et al. 2002; Staeva-Vieira et al. 2003). The point in prophase at which the defects block or delay the progression of meiosis differs from one organism to another, leading this checkpoint to be referred to in a general way as the recombination checkpoint. In *C. elegans* and mammals, the block in meiotic prophase induced by the recombination checkpoint is linked to the activation of apoptosis. For a recent review on the recombination checkpoint with the main focus on budding yeast, where it is best understood, see Hochwagen and Amon (2006).

Since meiotic recombination is initiated by programmed DNA damage, this checkpoint shares some components with the DNA damage checkpoint, as mentioned above (Lydall et al. 1996; Shimada et al. 2002). However, there are differences between the two checkpoints. Some of the proteins required for the mitotic DNA damage checkpoint are not required for the recombination checkpoint (Lydall et al. 1996). In addition, the recombination checkpoint uses meiosis-specific effector kinases (Higashitani et al. 2000; MacQueen and Villeneuve 2001; Perez-Hidalgo et al. 2003; Shimada et al. 2002; Xu et al. 1997). Finally, some of the cell cycle targets controlled by the two checkpoints are different or meiosis-specific (Chu and Herskowitz 1998; Hepworth et al. 1998; Leu and Roeder 1999; Tung et al. 2000). Interestingly, in mammalian meiosis the recombination checkpoint displays sexual dimorphism (Morelli and Cohen 2005). Although using the same components, the checkpoint in females is less effective than in males. This sexual dimorphism is also present in the SAC (Morelli and Cohen 2005). The relaxation of checkpoints in female mammals could explain the high frequency of defective oocytes produced in human meiosis.

As mentioned above for the SAC, the recombination checkpoint is also active in normal meiosis, and not only when synapsis or recombination are perturbed. The normal process of synapsis and recombination triggers checkpoint activation to ensure that the first meiotic division will not occur prematurely. Both in fission and budding yeasts, *spo11* mutants or mutants that do not produce recombinogenic DNA breaks are advanced in meiosis I entry (Hochwagen et al. 2005; Malone et al. 2004; Martin-Castellanos et al. 2005; Molnar et al. 2003; Wu and Burgess 2006).

5.1.1

Targets of the Recombination Checkpoint

How does the recombination checkpoint work? Most of our knowledge about the molecular mechanisms that lead to the meiotic block or delay comes

from work carried out in budding yeast and, more recently, in fission yeast. In budding yeast, the recombination checkpoint controls meiotic progression by two different mechanisms (Fig. 4: budding yeast). First, the activation of the checkpoint correlates with high levels of phospho-CDK at the catalytic site, and the deletion of *SWE1* (*WEE1*) or the expression of a non-phosphorylatable CDK version bypasses the pachytene arrest imposed by *zip1*, *dmc1*, and *hop2* mutants (Leu and Roeder 1999). Since Swe1/Wee1 is a kinase that inhibits cyclin/CDK complexes by direct phosphorylation during the mitotic cycle, it has been proposed that the same would apply for meiosis. In *Drosophila*, Wee1 could also be a checkpoint target. Wee1 is post-translationally modified and mislocalized in a Chk2-dependent manner when the checkpoint is activated in *spn-B* (*RAD51* homolog) mutants (Abdu et al. 2002). In addition to this mechanism, in budding yeast the checkpoint also controls the levels of cyclin Clb1 by regulating the activity of the meiosis-specific transcription factor Ndt80 (Chu and Herskowitz 1998; Hepworth

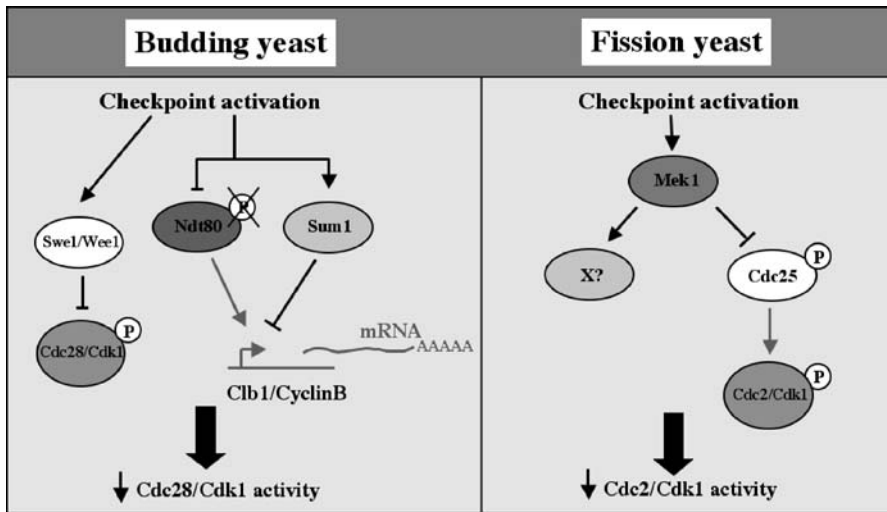


Fig. 4 Molecular mechanisms of the meiotic block or delay established by the recombination checkpoint. **Budding yeast** The recombination checkpoint controls meiotic progression by phosphorylation and inactivation of Cdc28 and by transcriptional repression of Clb1. Swe1/Wee1 is presumably the kinase involved in Cdc28 phosphorylation, since it is required for the pachytene arrest imposed by several mutants. Transcriptional repression of Clb1 is achieved by controlling the activity of the transcriptional activator Ndt80 (preventing its phosphorylation), and by controlling the activity of the transcriptional repressor Sum1 (increasing its level). **Fission yeast** The recombination checkpoint controls meiosis progression by phosphorylation and inactivation of Cdc2 in a Mek1-dependent manner. Mek1-dependent phosphorylation of Cdc25 is at least one of the mechanisms involved in the meiotic delay. *Black lines* indicate participation in the process. *Gray lines* indicate inhibition of such participation

et al. 1998; Tung et al. 2000). The checkpoint arrest can be bypassed by increasing the levels of Ndt80 or Clb1. The transcription factor accumulates in a phosphorylated form during prophase in a wild-type meiosis, but remains unphosphorylated in checkpoint-arrested cells (Leu and Roeder 1999; Tung et al. 2000). This has led to the proposal that Ndt80 must be phosphorylated in order to be active, and the checkpoint inhibition of this phosphorylation could be a mechanism to ensure the blockage of meiosis progression (Tung et al. 2000). In addition, the levels of the transcriptional repressor Sum1, which binds to some of the Ndt80-controlled promoters, are also regulated by the recombination checkpoint (Lindgren et al. 2000). The cooperation between the two mechanisms is seen in the fact that the double mutants *swe1 zip1*, *swe1 dmc1*, or *swe1 hop2*, although released from the pachytene arrest, are still delayed in meiosis I entry; only when Clb1 is expressed is the recombination checkpoint completely abolished and the cells progress into meiosis I with wild-type kinetics (Leu and Roeder 1999).

In fission yeast, the delay caused by the recombination checkpoint is established at least by controlling the phosphatase Cdc25 (Fig. 4: fission yeast). Checkpoint activation requires a meiosis-specific effector kinase, Mek1, and correlates with high levels of phospho-CDK in a Mek1-dependent manner (Perez-Hidalgo et al. 2003; Shimada et al. 2002). In addition, the checkpoint is unable to delay meiosis I entry in cells carrying a *cdc25* mutant version at several phosphorylation sites, the same sites that are required for the control of Cdc25 nuclear exclusion by the mitotic DNA damage checkpoint (Perez-Hidalgo et al. 2003). Cdc25 is the first component of the cell cycle machinery described to be phosphorylated in a checkpoint kinase-dependent manner in the meiotic checkpoint.

5.1.2

Chromosomal and Nucleolar Proteins in the Recombination Checkpoint

Interestingly, mutants that affect the meiotic chromosomal architecture (components of chromosomal axes such as Mek1, Red1, and Hop1) and mutants that presumably affect nucleolar function (*pch2*, *fpr3*) also bypass the pachytene arrest established by the recombination checkpoint in budding yeast (Hochwagen et al. 2005; Woltering et al. 2000; Xu et al. 1997). However, it is not clear what the function of these proteins is. Since the formation and resolution of the recombinogenic DSBs occurs in the context of chromosomes undergoing synapsis, it is possible that the chromosomal axis proteins might help to mark the sites of recombination, and in this way trigger checkpoint activation (Xu et al. 1997). In agreement with this, the Mek1 kinase (homolog to *S. pombe* Mek1) associates with and phosphorylates Red1 both in vitro and in vivo, although it has not been proven that it does so in a direct way, and when the checkpoint is activated cells accumulate a phosphorylated version of Red1 (Bailis and Roeder 1998; de los Santos and Hollingsworth

1999). The kinase activity of Mek1 is counteracted by the phosphatase Glc7 (Bailis and Roeder 2000). Conversely, it has been shown that the kinase activity of Mek1 depends on Red1 (Wan et al. 2004). Mek1 kinase activity is low in *red1* mutants and a phospho-Red1 protein binds to the FHA domain of Mek1, a conserved phosphoprotein binding domain. Moreover, the mutant version of Mek1 lacking the FHA domain behaves as a null mutant and is a less active kinase in vitro. The model proposes that Mek1-independent phospho-Red1 would function as a docking place for Mek1 loading to the sites of recombination and activation of kinase activity (Wan et al. 2004).

The role of the nucleolus in the recombination checkpoint is not well understood. Pch2 is a meiosis-specific ATPase that, when mutated, bypasses the pachytene arrest established by *zip1* mutants without restoring the primary synapsis defect of the *zip1* mutant. Pch2 protein localizes predominantly in the nucleolus, depending on the chromatin silencing proteins Sir2 and Dot1, and this nucleolar localization is required for normal checkpoint function (San-Segundo and Roeder 1999, 2000). These authors proposed that the nu-

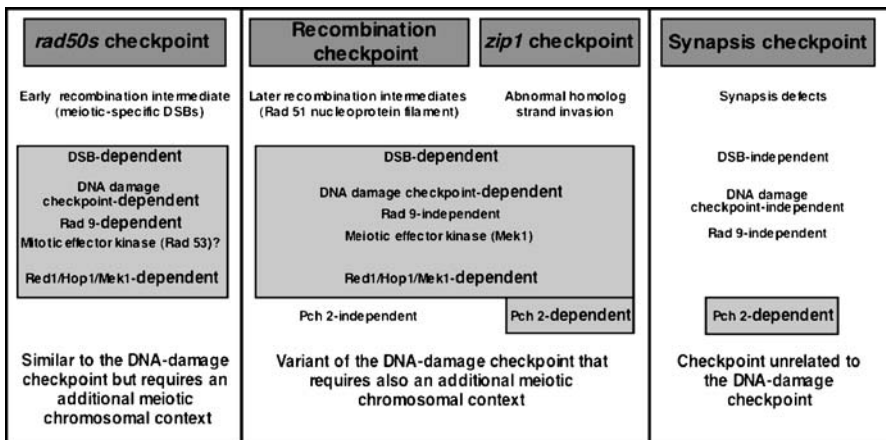


Fig. 5 Meiosis-specific checkpoints. Different checkpoints are described in regard to the signal that activates them and their genetic requirements. The genetic requirements are enclosed in *gray boxes*. The DSB-dependency is based on the *SPO11* (*rec12⁺*) requirement to establish the checkpoint. The *rad50S* checkpoint requires DSBs, components of the DNA-damage checkpoint involved in lesion recognition, the adaptor protein Rad9, probably the effector kinase Rad53, and a chromosomal context. The recombination checkpoint requires DSBs, components of the DNA-damage checkpoint but not the adaptor protein Rad9, the meiosis-specific effector kinase Mek1, and a chromosomal context. The *zip1* checkpoint requires all the genetic functions of the recombination checkpoint in addition to Pch2 (a nucleolar protein). The only genetic requirement known for the synapsis checkpoint is *pch-2*. It does not require components of the DNA-damage checkpoint involved in lesion recognition, and in budding yeast, nor the adaptor protein Rad9. The checkpoint has been described in *C. elegans* and budding yeast, and probably also exists in mammals

cleolus could titrate out factors required for the exit from pachytene arrest, as in the mitotic cycle the nucleolus has a function in mitosis exit by sequestering the Cdc14 phosphatase. The release of these factors in the *pch2* mutants would allow meiotic progression (Roeder and Bailis 2000). Interestingly, another protein required for pachytene arrest, Fpr3, has also been reported to localize in the nucleolus (Hochwagen et al. 2005). Fpr3 is a constitutively expressed proline isomerase required to maintain the checkpoint arrest established by *dmc1* mutants and to localize Glc7 in the nucleolus early on in meiosis. Both proteins interact physically and counteract each other in checkpoint-activated cells. However, it seems that Fpr3 and Pch2 do not function in the same way, since Fpr3 leaves the nucleolus as cells enter meiosis while Pch2 needs to be present in the nucleolus to exert its checkpoint function (Hochwagen et al. 2005).

In both budding and fission yeasts the bypass of meiotic arrest or delay imposed by different mutants depend on different genetic functions (Hochwagen et al. 2005; San-Segundo and Roeder 1999; Shimada et al. 2002; Zierhut et al. 2004). These observations indicate that the so-called recombination checkpoint could indeed be a mixture of different cellular responses. Hochwagen and Amon (2006) have classified the recombination checkpoint in *S. cerevisiae* into different categories (Fig. 5): the *rad50S* checkpoint, which responds to early meiosis-specific recombination intermediates and requires both the adaptor protein Rad9 and the chromosome structure proteins Red1/Hop1/Mek1; the recombination checkpoint itself, which responds to later recombination intermediates and requires chromosomal proteins but not Rad9; and the *zip1* checkpoint, which in addition to the chromosomal proteins requires Pch2.

5.2

The Synapsis Checkpoint

In budding yeast and mammals, the recombination checkpoint is activated in response to defects in both synapsis and recombination, since in these organisms synapsis and recombination are linked. However, in other organisms, such as *Drosophila* and *C. elegans*, this is not the case, and these two processes can occur independently of each other (Zickler 2006). Interestingly, recent work on *C. elegans* has shown that defects in synapsis lead to the activation of a genetically independent checkpoint pathway (Bhalla and Dernburg 2005; reviewed by Meier and Gartner 2006). In *C. elegans*, each chromosome has a unique region, the pairing center, that promotes pairing and synapsis. Using hemizygous and homozygous pairing center mutants for the X chromosome, the authors found that in both situations apoptosis was increased and was correlated with defects in synapsis. However, only in the hemizygous situation was apoptosis blocked by a mutation in *pch-2* (the *C. elegans* homolog of budding yeast *PCH2*), where mutations that block DSB forma-

tion or the DNA damage checkpoint response did not have an effect on the apoptosis observed in the hemizygous situation. On the other hand, the apoptosis due to the homozygous pairing center defect was only abolished by mutations that block DNA break formation and checkpoint function, but not by *pch-2* mutation. When apoptosis was blocked in either situation (hemi- or homozygous for the pairing center mutation), the percentage of oocytes with achiasmatic X chromosomes, and therefore the male (X0) incidence, increased. Thus, two situations leading to unsynapsed X chromosomes activate two different meiotic checkpoints: the DNA damage checkpoint and a new checkpoint, the synapsis checkpoint (Fig. 5). It has been proposed that besides PCH-2 the synapsis checkpoint requires the pairing center binding protein HIM-8 (Bhalla and Dernburg 2005).

Pch2 in budding yeast localizes predominantly in the nucleolus and this localization is required for its function in the pachytene checkpoint (see above). Since the nucleolus contains ribosomal DNA repeats that remain largely unsynapsed in pachytene, Pch2 could mark these chromosomal positions. Consistent with this idea, the lack of *PCH2* in budding yeast increases recombination in ribosomal DNA and recruitment to the nucleolus of the chromosomal protein Hop1 (San-Segundo and Roeder 1999). It therefore seems worth analyzing the localization of PCH-2 in *C. elegans*.

In budding yeast it was not clear until very recently whether a synapsis checkpoint could indeed exist. *spo11* mutants that do not produce DSBs and that are severely affected in synapsis, due to the coupling of these two processes in this yeast, are not delayed or arrested in prophase but are accelerated (Hochwagen et al. 2005; Malone et al. 2004). Nevertheless, the pachytene arrest established by the loss of the helicase Sgs1 is not overcome by deletion of *SPO11* (Rockmill et al. 2003), indicating that a DSB-independent checkpoint could also exist in budding yeast. Interestingly, a recent report has shown that, although depending on DSB formation, a genetically different pathway is required to respond to incomplete synapsis, and this pathway requires *PCH2* (Wu and Burgess 2006). In fission yeast, SC is not formed, and therefore proper synapsis is not observed (Loidl 2006). However, homologous chromosomes are paired. *bqt2* mutants that are impaired in chromosome pairing and not in DSB formation are not delayed in prophase, which argues against a pairing checkpoint in fission yeast (Davis and Smith 2006; Martin-Castellanos et al. 2005). The fact that deletion of *rec12*⁺ does not overcome the prophase delay of *dmc1* mutants (Shimada et al. 2002) points to the existence of a DSB-independent checkpoint, as in budding yeast.

It is possible that in mammals both checkpoints (recombination and synapsis) also exist, and that primary defects in synapsis also lead to checkpoint activation, although in this case the same checkpoint pathway is probably used. Mammalian DNA damage checkpoint proteins, such as RAD1, ATR, TopBP1, BRCA1, and CHK1, have been observed to coat unsynapsed chromosomes and/or the axes of the XY chromosomes (Flaggs et al. 1997; Freire et al.

1998; Keegan et al. 1996; Moens et al. 1999; Perera et al. 2004; Turner et al. 2005). At least in the case of ATR and RAD1, these proteins do not colocalize with foci of RAD51, a marker for recombination sites, suggesting that unsynapsed chromosomes are detected independently of recombination (Freire et al. 1998; Moens et al. 1999). Moreover, coating of the unsynapsed chromosomes with checkpoint proteins is temporally different from the chromosome coating in response to recombination (Turner et al. 2005). Finally, unsynapsed sex chromosomes can activate the meiotic checkpoint in the mouse (Odorisio et al. 1998), and elimination of the SPO11 endonuclease does not completely eliminate spermatocyte apoptosis or oocyte loss in checkpoint-compromised meiosis, suggesting the activation of an additional checkpoint (Di Giacomo et al. 2005). Recently, a compelling amount of evidence indicated that defects in synapsis induce transcription inactivation of the unsynapsed chromosome regions, suggesting that in addition to checkpoint activation asynapsis could contribute to meiotic arrest through the silencing of genes that are crucial for meiosis (Baarends et al. 2005; Turner et al. 2005).

6

Conclusions and Future Directions

In the last few years our knowledge has increased on how the basic cell cycle machinery is modulated to achieve the variations that a meiotic division requires. Meiosis runs with a specific transcriptional program, and uses meiosis-specific cell cycle regulators in addition to the ones already used in vegetative growth. However, apart from the recombination checkpoint, very little is known about how the progression through the meiotic program is coordinated with key features, such as DSB formation, chromosome pairing, or SC formation. How do the cyclin/Cdk complexes impinge on these processes? It is reasonable to think that elements involved in these processes could be targets of CDK activity; moreover, in mammals, CDK2 has been shown to decorate meiotic chromosomes. Recently, it has been described that CDK activity is required to phosphorylate a protein involved in DSB formation in budding yeast. This pioneer study opens a new focus of research in the meiotic field. An important goal for the future will be to translate all this basic information into the clinic, in order to understand how infertility problems arise in the light of all these basic findings.

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